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October 15, 1997

Dr. C. W. Jameson
National Toxicology Program
Report on Carcinogens
Building 4401 Room 3127
79 Alexander Drive
Research Triangle Park, NC 27709

Dear Doctor Jameson:

Enclosed are 15 copies of a submission for review by the National Toxicology Program in connection with its consideration of the listing of "smokeless tobacco" in the "Report on Carcinogens, Ninth Edition." You have previously advised this firm that the submission of this material by mid-October would enable its consideration during the meeting scheduled for the end of this month.

Please contact the undersigned if you have questions about the materials.

Sincerely,

Stuart M. Pape

SMP:ro286765

Enclosures

National Toxicology Program
Review of Smokeless Tobacco
for Possible Listing in the
Ninth Report on Carcinogens

Comments of

Conwood Company, L.P.
National Tobacco Company, L.P.
Swedish Match North America Inc.
Swisher International, Inc.
United States Tobacco Company

October 14, 1997

Comments of

Conwood Company, L.P.
National Tobacco Company, L.P.
Swedish Match North America Inc.
Swisher International, Inc.
United States Tobacco Company

regarding

National Toxicology Program Review of Smokeless Tobacco
for Possible Listing in the Ninth Report on Carcinogens

The above-listed manufacturers of smokeless tobacco products -- Conwood Company, L.P., National Tobacco Company, L.P., Swedish Match North America Inc., Swisher International, Inc., and United States Tobacco Company (the "smokeless tobacco manufacturers") -- submit the following comments to the National Toxicology Program ("NTP") in connection with its review of smokeless tobacco for possible listing in the Ninth Report on Carcinogens as either "known to be a human carcinogen" or "reasonably

anticipated to be a human carcinogen," as announced in a Federal Register Notice of July 11, 1997 (62 Fed. Reg. 37272).[1]

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[1] The smokeless tobacco manufacturers understand that NTP's criteria for classifying substances as either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens" are as follows:

1. Known to be Human Carcinogens:

   There is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer.

2. Reasonably Anticipated to be Human Carcinogens:

   There is limited evidence of carcinogenicity from studies in humans which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded; or

   There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposure, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset; or

   There is less than sufficient evidence of carcinogenicity in humans or laboratory animals, however, the agent, substance or mixture belongs to a well defined, structurally-related class of substances whose members are listed in a previous Annual or Biennial Report on Carcinogens as either a known to be human carcinogen, or reasonably anticipated to be human carcinogen or there is convincing relevant information that the agent acts through mechanisms indicating it would likely cause cancer in humans.

The smokeless tobacco manufacturers have requested several experts in the fields of epidemiology, toxicology and experimental carcinogenesis to review the relevant epidemiological and experimental data published in the scientific literature, and to provide statements to NTP summarizing that data and expressing their expert opinions as to whether that scientific data would support the classification of smokeless tobacco, under NTP's criteria, as either "known to be a human carcinogen" or "reasonably anticipated to be a human carcinogen."

Dr. Kenneth D. MacRae, a Reader in Medical Statistics at the Imperial College School of Medicine of the University of London, a Fellow of the Royal Statistical Society and a member of the editorial committee of the British Medical Journal, has reviewed the epidemiological data relating to smokeless tobacco and cancer. Dr. MacRae's analysis is annexed as Attachment A. His conclusion is as follows:

> It is my opinion that the epidemiological data do not support the conclusion that smokeless tobacco has been shown to be a cause of cancer in humans. Nor, in my opinion, does the epidemiological data, taken as a whole, support a finding that the use of smokeless tobacco is a risk factor for oral cancer. Accordingly, I do not believe that the epidemiological data support NTP's classifying smokeless tobacco among substances that are either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens."[2]

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[2] Submission of Kenneth D. MacRae, Ph.D. to NTP dated October 10, 1997 at pages 2-3.

Prof. Paul Grasso and his colleagues at the School of Biological Sciences, University of Surrey (U.K.) have conducted an extensive review of the scientific literature on smokeless tobacco in order to examine the experimental evidence relating to smokeless tobacco and cancer. Their analysis of the experimental data has been compiled in a monograph entitled "Assessment of the Experimental Evidence Relating to Smokeless Tobacco and Oral Cancer," issued on October 31, 1996. A copy of the monograph is annexed as Attachment B. Based on that assessment and their continuing review of the relevant experimental data published in the scientific literature, Prof. Grasso and his colleagues have concluded that:

> Overall, the experimental studies support the conclusion that smokeless tobacco is not carcinogenic in the oral cavity of laboratory animals. Moreover, it is our judgment that those experimental studies do not support the listing of oral use of smokeless tobacco products in the NTP's Ninth Report on Carcinogens.[3]

Dr. Torbjorn Malmfors, an internationally noted toxicologist and past president of the Swedish Society of Toxicology, has also reviewed the published experimental data regarding the possible carcinogenicity of smokeless tobacco. His analysis is annexed as Attachment C. Dr. Malmfors' conclusion is as follows:

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[3] Letter from Professor Paul Grasso and his colleagues to NTP dated October 6, 1997, a copy of which is annexed as part of Attachment B.

> There has been a substantial number of animal studies performed to investigate the possible carcinogenicity of smokeless tobacco. Studies have been performed in mice, hamsters, rats and monkeys. Different modes of exposure have been employed for a life span period in most instances. A maximally possible amount of smokeless tobacco has been administered in most experiments both systemically and locally. The animal strains used and the most relevant sites have all been shown to be sensitive to various experimental carcinogens.
>
> * * *
>
> My overall evaluation of the animal studies is that there are no results which are sufficiently significant, valid, or relevant to human beings according to scientific principles, to justify a conclusion under the criteria established by NTP that smokeless tobacco is "reasonably anticipated to be a human carcinogen."[4]

Based upon the analyses of the epidemiological and experimental data relating to smokeless tobacco and cancer submitted by Dr. Kenneth D. MacRae, Professor Paul Grasso and his colleagues and Dr. Torbjorn Malmfors, the smokeless tobacco manufacturers submit that NTP should not classify smokeless tobacco as either "known to be a human carcinogen" or "reasonably anticipated to be a human carcinogen."

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[4] Submission of Torbjorn Malmfors, M.D., Ph.D. to NTP dated October 10, 1997 at p. 19.

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Kenneth D. MacRae MA PhD FIS
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10 October, 1997

Dr. C.W. Jameson
National Toxicology Program
Report on Carcinogens
MD WC-05, PO Box 12233
Research Triangle Park, NC 27709

Re: NTP Review of Smokeless Tobacco for Possible Listing in the Ninth Report on Carcinogens

Dear Dr. Jameson:

I have been requested by several manufacturers of smokeless tobacco products to comment on the epidemiological data relating to the issue of whether smokeless tobacco products can be classsified pursuant to the criteria established by the National Toxicology Program as being either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens." My analysis and conclusions are attached.

Very truly yours,

Kenneth D. MacRae

Kenneth D. MacRae

SUBMISSION BY KENNETH D. MACRAE, Ph.D. RELATING TO THE NATIONAL TOXICOLOGY PROGRAM'S REVIEW OF SMOKELESS TOBACCO FOR POSSIBLE LISTING IN THE NINTH REPORT ON CARCINOGENS

I am Dr. Kenneth D. MacRae, a medical statistician, and am currently Reader in Medical Statistics at the Imperial College School of Medicine of the University of London, England. From 1984 to 1997 I was Reader in Medical Statistics at the Charing Cross and Westminster Medical School, which became part of the Imperial College School of Medicine in 1997. I completed my undergraduate and postgraduate studies at the University of Aberdeen in Scotland, receiving my Ph.D. in Statistical Decisions in 1970. From 1969 to 1976 I was Lecturer in Medical Statistics at the Queen's University of Belfast. From 1976 to 1984 I was Senior Lecturer in Medical Statistics at the Charing Cross Hospital Medical School of the University of London, England, until I assumed my current position. I am a Fellow of the Royal Statistical Society and a member of the editorial committee of the British Medical Journal.

I have been the statistician responsible for the design and analysis of several multi-centre trials in the field of cancer therapy, all of which have received external funding, mainly from the British Cancer Research Campaign, but including one trial funded by the N.C.I. I have had a particular interest in the validity of epidemiological research and on

specific issues arising out of epidemiological studies. My full curriculum vitae and list of publications are attached.

I have been asked by several manufacturers of smokeless tobacco products to comment upon the epidemiological data relating to the issue of whether smokeless tobacco products can be classified pursuant to the criteria established by the National Toxicology Program as either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens."

I understand that NTP's criteria for listing agents, substances or mixtures as "known to be human carcinogens" are as follows:

> "There is sufficient evidence of carcinogenicity from studies in humans which indicates a causal relationship between exposure to the agent, substance or mixture and human cancer."

I further understand that NTP's criteria for listing agents, substances or mixtures as "reasonably anticipated to be human carcinogens," as the criteria relate to epidemiological studies, are as follows:

> "There is limited evidence of carcinogenicity from studies in humans which indicates that causal interpretation is credible but that alternative explanations such as chance, bias or confounding factors could not adequately be excluded."

It is my opinion that the epidemiological data do not support the conclusion that smokeless tobacco has been shown to be a cause of cancer in humans. Nor, in my opinion, does the epidemiological data, taken as a whole, support a finding that the use of

smokeless tobacco is a risk factor for oral cancer. Accordingly, I do not believe that the epidemiological data support NTP's classifying smokeless tobacco among substances that are either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens."

Although the IARC (1985) and Surgeon General's (1986) reports regarding smokeless tobacco and cancer reviewed many epidemiological studies, the data are both inconsistent and quantitatively and qualitatively sparse. Many of the studies fail to control for one or more confounding factors. Some involve study populations that are small or fail to report sufficient information so that it is difficult to perform any meaningful statistical analyses of the data. I have personally examined these and subsequent epidemiological studies, as well as the doctoral thesis and data underlying the retrospective case control study by Winn, et al. (1981), the principal study relied upon by both the IARC and the Surgeon General.

Epidemiology is the study of disease by statistical methods in order to identify factors which are statistically associated with the occurrence of disease. It does not deal with the mechanisms or pathogenesis of disease. Statistical association alone does not establish a causal relationship; it does form the basis for further laboratory and clinical research. There are several types of epidemiological studies, and they, as might be expected, vary greatly in their strengths and susceptibilities to bias.

The epidemiological data with respect to smokeless tobacco fall into three categories: case series, retrospective case control studies, and prospective cohort studies. As you know, case series cannot even establish a "statistical association" due to the absence of controls. At best, they create an interest in the possibility of a relationship between a disease and some factor. A search for possible statistical associations between a disease and one or more factors, however, can be carried out by using one of two epidemiological study designs, namely, the retrospective case control design and the prospective cohort design. Using these designs, statistical associations with oral cancer have been reported for the following with varying degrees of consistency: diet, mouthwash, alcohol consumption, smoking, oral hygiene, ill-fitting dentures, anaemia, Plummer-Vinson disease, occupation, vitamins, leukoplakia, syphilis, Epstein-Barr virus, gender, socio-economic status, age, race, and ethnicity.

In addition, smokeless tobacco, the present subject of inquiry, has also been reported to be statistically associated with oral cancer in some studies. With respect to smokeless tobacco and oral cancer, the best retrospective case control study to date is the Winn study. Yet, even this study has admitted "methodological limitations" and cannot

provide the basis for a conclusion that smokeless tobacco causes cancer. A brief review of the epidemiological studies relating to smokeless tobacco and oral cancer follows.[1]

While some studies report a statistical association between the use of smokeless tobacco and oral cancer, others have not. At the outset, it should be reiterated that virtually all these studies suffer from various methodological weaknesses, such as very limited populations in confined geographic regions or failure to account for confounding factors. Nevertheless, a review of these studies is useful to demonstrate the equivocal nature of the data on the issue of statistical association.

The major epidemiological studies showing no association between smokeless tobacco use and oral cancer include: Smith, et al. (1970), Smith (1975), Wynder, et al. (1957), Peacock, et al. (1960), Martinez (1969), Wynder and Stellman (1977), Browne, et al. (1977), , Young, et al. (1986), Sterling, et al. (1992), Mashberg, et al. (1993) and Muscat, et al. (1996).

Smith, et al. (1970) and Smith (1975) report a prospective follow-up study conducted in Tennessee involving 15,000 smokeless tobacco users, 1,550 of whom were followed for 10 years. It was reported that none of these individuals developed either oral

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[1] As to epidemiological data relating to any possible association between smokeless tobacco and cancers outside the oral cavity, the 1986 Surgeon General's report stated: "Evidence for an association between smokeless tobacco use and cancers outside of the oral cavity in humans is sparse. Some investigations suggest that smokeless tobacco users may face increased risks of tumors of the upper aerodigestive tract, but results are currently inconclusive" (p. xxiii). I am not aware of any studies published over the past 11 years which would warrant a change in that conclusion.

cancer or serious changes in the oral mucosa. The primary researcher concluded: "I believe that the type of snuff used in this country cannot logically be considered as carcinogenic in view of the large number of patients who have used snuff for many years with no clinical or histological evidence of tissue change."

Wynder, et al., (1957) reported no statistically significant association between tobacco chewing and oral cancer in a large Swedish population. Peacock, et al. (1960) in a retrospective study of 45 oral cancer patients reported no statistically significant association between oral cancer and smokeless tobacco use except in individuals from a low economic status who were over 60 years of age. Information on alcohol use or cigarette smoking was not obtained in sufficient detail to be accounted for in the statistical analysis.

Martinez (1969), in a retrospective study of 400 cases and 1,200 controls, found that "[p]atients with cancer of the mouth did not often use chewing tobacco disproportionately...." Wynder and Stellman (1977), in a retrospective study at 20 hospitals in 8 American cities, reported on 873 cases of oral cancer and found the use of smokeless tobacco in these cases was almost identical to that of the controls.

Browne, et al. (1977) conducted a retrospective study in England of 75 oral cancer patients and 150 controls and concluded that "[t]obacco chewing, which was restricted to miners, was equally common (45%) in the two groups." Young, et al. (1986) was a retrospective case control study of 623 Wisconsin patients with cancer of the head and

neck. With respect to the patients who ever used snuff or chewed tobacco regularly, it was found that there were no statistically significant differences between cases and controls.

Sterling, et al. (1992) used the National Mortality Followback Survey and the National Health Interview Survey to compute risk estimates for mortality for all cancer, oral cancer and cancer of the digestive organs for use of smokeless tobacco, controlling for potential confounding from smoking, drinking alcohol and occupation. No statistically significant associations were found for any of these groups of cancer.

Mashberg, et al. (1993) conducted a hospital based case-control study of 359 patients with oral cavity - oropharynx cancer and 2,280 controls. No increased risk of oral cancer was found for use of snuff or chewing tobacco.

Muscat and his colleagues at the American Health Foundation (including Dr. Ernst L. Wynder) conducted a hospital based case-control study of 1009 patients with oral cancer and 923 age-matched controls (Muscat, et al. (1996)). They found that oral snuff use and use of chewing tobacco were unrelated to oral cancer, 1.3% of male cases and 1.6% of male controls having used snuff at least once a week for one or more years, and 5.5% of male cases and 5.3% of male controls having used chewing tobacco at least once a week for more than one year. Among women, only 2 cases and 1 control reported snuff use, and no women reported using chewing tobacco regularly.

The major epidemiological studies reporting statistical associations of varying strengths between smokeless tobacco and oral cancer, include: Moore, et al. (1953), Vogler, et al. (1962), Vincent and Marchetta (1963), Williams and Horm (1977), Westbrook, et al. (1980), Stockwell, et al. (1986), Spitz, et al. (1988), and Winn, et al. (1981). Moore, et al. (1953) was a retrospective case control study of 40 cases of mouth cancer in Minnesota. The authors report a statistically significant association between smokeless tobacco use and mouth cancer. The data reflect a relative risk of 4.0 for this association. The 1986 Surgeon General's Report discounts this study as a whole because the relative risk estimates for the association between smoking and mouth cancer were less than 1.0 (0.6 for pipe smoking; 0.54 for cigarette and cigar smoking).

Vogler, et al. (1962) in a retrospective study looked at 333 cases of cancer of the mouth, pharynx and larynx and reported a statistically significant association with smokeless tobacco use. The study, however, does not take account of alcohol and smoking as confounding factors.

Vincent and Marchetta (1963) reported a retrospective study of 106 patients with cancer of the head and neck and 150 controls. The study reported a higher rate of usage of smokeless tobacco in male patients with oral cavity cancer than in controls. This comparison was based on only 9 smokeless tobacco using cases and 5 smokeless tobacco using controls. Therefore broad conclusions from these limited data cannot be drawn. In addition, this study failed to control for smoking, alcohol use, and age.

Williams and Horm (1977) did a case control analysis of cancers based on data from the Third National Cancer Survey. The statistically significant associations reported in this study between smokeless tobacco use and oral cavity cancer in males but not in females were based on only 11 males who were smokeless tobacco users. Moreover, this study used other cancer sites as controls and as the authors concede "[t]he intercancer comparison approach required for the analysis of these data is one of the most likely sources for possible misinterpretations.... This approach can produce factitious inverse (or even positive associations)...."

The medical records of 55 female patients treated at the University of Arkansas with cancer of the alveolar ridge or buccal mucosa were compared with the records of 55 randomly selected female hospital controls (Westbrook, et al., (1980)). Fifty of the cases and only 1 of the controls were reported to be snuff dippers. The results of this study appear to be grossly out of line with any other reported study as the data reflect a relative risk of 540.0. These extraordinary results are an extreme example of the methodological problems inherent in retrospective case control studies. Here, snuff usage information for the cases and controls was obtained from the medical records of the patients, and many of the controls were undoubtedly being treated for conditions that would not elicit information relating to snuff use. As the Surgeon General's Report observed with regard to this study, "[n]o reliable estimates of risk can be derived from this study because of the

strong possibility that there was not comparable elicitation of exposure information for cases and controls."

Stockwell, et al. (1986) conducted a retrospective case control study involving 2,351 cases of head and neck cancer in Florida and reported an 11-fold risk of mouth and gum cancer in smokeless tobacco users. As the authors themselves noted, there was no control for confounding by smoking and alcohol use. In addition, as with Williams and Horm (1977), the controls were other cancer patients.

Spitz, et al. (1988) reported on a retrospective case control study in Texas of 185 patients with cancer of the larynx, tongue, orohypopharynx, and oral cavity. Although the authors report that a relative risk of 3.4 is statistically significant, based on 9 snuff dipping cases (4 larynx, 4 oral cavity and 1 orohypopharynx) and 4 snuff dipping controls, in fact the odds ratio is actually 2.3 and is not statistically significant. All 9 cases drank alcohol and smoked, meaning that the association with snuff dipping is completely confounded by smoking and drinking. In addition, when analyzed by site, the data show no association between oral cavity cancer and snuff use.

The best retrospective case control study to date reporting an association between smokeless tobacco use and oral cancer is Winn, et al. (1981), but this study also has methodological limitations, which are recognized by the authors, and does not establish that smokeless tobacco is a risk factor for oral cancer, much less that it is a cause of oral cancer.

This was a retrospective case control study involving 232 women in North Carolina with oral and pharyngeal cancer selected from hospital records and death certificates and 410 controls that were matched for age, race, source of ascertainment (i.e., hospital or death certificate), and county of residence. Interviews were conducted with the subjects or, where the subjects were unavailable, with next-of-kin to accumulate information about the cases and controls with respect to a broad range of topics. The proportion of next-of-kin interviews was much greater for cases (51%) than for controls (21%) in the hospital series. The hospital series constituted the greater proportion of the study subjects. The death certificate series was of course all next-of-kin interviews.

A major methodological problem, therefore, with this study is that substantially more next-of-kin interviews were conducted with cases than with controls. Analysis of the interview technique reveals that next-of-kin reported more snuff usage than the study subject herself. Therefore, the interview source itself may have increased the snuff usage among the cases in this study compared to the controls thereby inflating rates or relative risk as to snuff use.

Next, Winn, et al. is repeatedly cited, including by the IARC and the Surgeon General, for their reported finding that snuff dipping for greater than 50 years resulted in a 47.5 relative risk for gum and buccal cancer. It should be noted that this relative risk is out of line with virtually every other study on this subject. The researchers have achieved

this extraordinary relative risk by excluding from their calculations the strata of the data which do not show statistically significant associations.

For example, pharynx and other mouth sites do not show a statistically significant risk, so the analysis is confined to the gum and buccal sites; smokers do not show a significant association, so the analysis is confined to non-smokers; and the death certificate cases do not show a statistically significant association, so the analysis is confined to the hospital cases. They also chose periods of duration of use (i.e., 1-24 years, 25-49 years, 50 and over years) in order to maximize the relative risk. However, when all cases and all controls for all sites are considered, the relative risk for 50 years or more of snuff dipping is actually 1.99.

In any event, even if the obviously biased selection of the data were appropriate, Winn, et al. used an inappropriate method for calculating the 50 years and over odds ratio because it ignores the matching of the cases and controls. If other more appropriate methods designed to take account for matching were used, the relative risk for this highly selected data would be approximately 5.

Winn, et al. also report a dose response relationship based on the same highly selective data discussed above. Yet, as Winn states in her doctoral thesis, when the data are taken as a whole, "[o]ral cancer was not clearly related to the amount or duration of snuff use."

Winn, et al. find that among those subjects who both smoked and dipped snuff, "the risk of oral and pharyngeal cancer was not exceptional," the relative risk being 1.2. Yet, snuff dipping in terms of can years in this group was greater than in non-smoking dippers who had a relative risk of 3.6. In addition, the smoking dippers had an average consumption of 15 cigarettes per day and 22 pack years while the non-smoking dippers obviously had no smoking exposure at all. These data, coupled with the absence of a dose response relationship, argues against the biological plausibility of the sometimes reported association between oral cancer and snuff use as being one of cause and effect.

A curious feature of this study is the absence of an association between oral cancer and snuff use in blacks. Another curious feature of this study is that the data show statistically significant negative associations between alcohol consumption and oral cancer in non-smokers and in dippers. Such anomalous results further illustrate the degree of caution that needs to be exercised in interpreting statistical associations whether positive or negative in a retrospective case control study such as this.

Finally, even if the Winn study was beyond reproach methodologically, it must be remembered that it is only one retrospective case control study with all the limitations inherent in such studies and that the study population to which it refers is hardly representative of the U.S. population in general, being largely elderly women from the Piedmont region of North Carolina. Broad conclusions can hardly be based on such a study. It is noteworthy that 16 years after the publication of the Winn study, which

garnered widespread attention in the public health community, the results of this study have not been reproduced in the Piedmont region of North Carolina, nor in any other population.

In direct contrast to her positive findings in non-smoking dippers in her case control study, Winn, et al. (1982) found no association with oral and pharyngeal cancer in non-smoking users of smokeless tobacco in a follow-up of 300,000 U.S. veterans from the Dorn study.

In summary, the statistical studies conducted to date do not convincingly show the use of smokeless tobacco to be a risk factor for oral cancer, let alone a cause of that disease. Accordingly, in my opinion, it would be unwarranted for the NTP to list smokeless tobacco products as either "known to be human carcinogens" or "reasonably anticipated to be human carcinogens."

Kenneth D. MacRae, Ph.D.

10 October, 1997

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Date and Place of Birth

27 October, 1942. Forres, Morayshire, Scotland, U.K.

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Education

School

Forres Academy 1948-1960. Scottish Leaving Certificate Higher Grades: English, Mathematics, Science (Physics and Chemistry), French and Latin.

Undergraduate Studies

University of Aberdeen 1960-1964.
Ordinary degree subjects: Mathematics,Physics, Chemistry and Biology.
Honours subject: Experimental Psychology.
Class prizes and first-class course certificates: Physics and Chemistry (1st Year), Mathematics (2nd Year), Psychology (3rd Year).
M.A. (2nd Class Honours in Psychology) 1964.

Postgraduate Studies

University of Aberdeen 1964-65. Course work: Mathematical Statistics and Biometry (Department of Statistics).

University of Aberdeen 1965-1967. Research work: Sequential Statistical Decision Making (Departments of Psychology and Statistics).
Ph.D. 1970.

Posts Held

1965-1967: Assistant Lecturer, Department of Psychology, University of Aberdeen. (Professor E.D. Fraser).

1967-1969: Lecturer, Department of Psychology, The Queen's University of Belfast. (Professor G. Seth).

1969-1976: Lecturer, Department of Medical Statistics, The Queen's University of Belfast. (Professor E.A. Cheeseman).

1976-1984: Senior Lecturer in Statistics, Charing Cross Hospital Medical School, University of London. (Attached to the Department of of Medicine, Professor A. Guz).

1984- : Reader in Medical Statistics, Charing Cross and Westminster Medical School, University of London (Attached to the Department of Medicine, Professor A. Guz, until 1996; Department of Public Health, Professor RDT Farmer from 1996. From 1 August 1997 the Medical School became part of Imperial College of Medicine, Science and Technology).

Professional Qualification

Fellow of the Institute of Statisticians, 1983.

Learned Societies

Fellow of the Royal Statistical Society, 1970.
Member of the Society for Social Medicine, 1975.
Member of the Medico-Legal Society, 1984.

Teaching Experience

1965-1967 (Aberdeen Department of Psychology): Statistics tutorials, practical course on experimental design, and advice on design and analysis of undergraduate and postgraduate research projects.

1967-1969 (Belfast Department of Psychology): Lecture course on analysis of variance and design of experiments. Lecture course in mathematical psychology. Supervision of practical course in experimental psychology, and of undergraduate thesis work. Advice on design and analysis for staff and postgraduate students in the department.

1969-1976 (Belfast Department of Medical Statistics): Lecture courses on statistics to undergraduate medical students, and for postgraduate courses for the Diploma in Public Health and the Diploma in Dental Public Health. Course in experimental design for the M.Sc. in clinical psychology. Occasional 'open' courses in statistics for medical school and hospital staff. Advisory service in design and analysis for medical school and hospital staff.

1976- (Charing Cross Hospital Medical School): Lecture courses on statistics, clinical biometry and epidemiological methods to

undergraduate medical students. 'Open' courses for medical school and hospital staff. Courses for postgraduate medical qualifications - M.R.C.Psych. (N.W. Thames Regional Course), the Diploma in Pharmaceutical Medicine and the Diploma in Clinical Science (organised by University College Cardiff, University of Wales). M.R.C.O.G. Course, Queen Charlotte's Hospital. The Advanced Course in Obstetrics and Gynaecology, Queen Charlotte's Hospital. F.R.C.R. in Clinical Oncology London Course. Assistance with projects for the M.Sc. in Statistics (Imperial College, University of London).

Advisory service in design and analysis for medical school and hospital staff.

Multi-Centre Clinical Trials

1970-1976: Ovarian Cancer Clinical Survey. (Chairman Dr G.A. Edelstyn, Northern Ireland Radiotherapy Centre).

1972-1978: Cancer Research Campaign Breast Trial. (Joint Chairmen Professor J.G. Murray, King's College Hospital Medical School, and Professor J. Mitchell, University of Cambridge).

1974- : Multicentre Cancer Chemotherapy Group. (Chairman Dr. G.A. Edelstyn, followed by Dr M.F. Spittle, The Middlesex Hospital)

1981- : Cancer Research Campaign Adjuvant Breast Trials (Chairman Professor M. Baum, King's College Hospital Medical School).

1982- : Multicentre CEA - Second-Look Surgery Colorectal Cancer Trial (Chairman Sir William Slack, The Middlesex Hospital, followed by Mr J.M.A. Northover, St Mark's Hospital).

1982- : Cancer Research Campaign Malignant Obstructive Jaundice Trials (Chairman Dr P.B. Cotton, The Middlesex Hospital, followed by Mr R.C.G. Russell, The Middlesex Hospital).

1983- : International Biliary Study Group (Chairman Professor Dr M. Classen, Frankfurt).

1988- : U.K. Hepatic Artery Pump Trial (Chairman Mr T.G. Allen-Mersh, The Westminster Hospital).

Multi-Centre Epidemiological Project

1984-92 : European Economic Community Study of the Adverse Effects of Alcohol Consumption in Pregnancy, including the Foetal Alcohol Syndrome - EUROMAC. (Chairman Professor C. du V Florey,Dundee).

Committees and Administration

1969-1976 (Queen's University, Belfast): Science Faculty Working Party on Student Intake. Medical Faculty Working Party on First-Year Exemptions. Medical Faculty Working Party on Regular Recurrent Expenditure and the Equipment Fund. The Inter-Faculty Committee on the Teaching of Computing.

1974-1976: Honorary Secretary of the Northern Ireland Branch of the Royal Statistical Society.

1975-1976: Honorary Treasurer of the Queen's University Staff Club.

1974-1978: Council Member, Action Cancer Northern Ireland.

1976-1980: Scientific Advisory Committee, Action Cancer.

1976-1982: Editorial Board of the British Journal of Nutrition.

1982-1983: President of the Charing Cross Hospital Medical School
1987-1988: Branch of the Association of University Teachers.

1985-88 : Member of the Ethical Committee of Charing Cross Hospital.
1988 : Vice-Chairman of the Ethical Committee.
1988-91 : Chairman of the Ethical Committee.

1986- : Member of the Scientific Advisory Board of the Smokeless Tobacco Research Council.

1987- : Member of the Scientific Advisory Committee of the Myofascial Pain Research Trust.

1987-90 : Secretary of the University of London Special Advisory Committee on Medical Statistics and Biometry.

1988- : Member of the Scientific Advisory Committee of the Cancer Research Campaign's Clinical Trials Centre.

1989-92 : Member of the Events Committee of the Institute of Statisticians.

1989- : Member of the Clinical Research Ethics Committee of the Royal College of General Practitioners.

1990- : Member of the Editorial Board of Statistical Methods in Medical Research.

1992- : Member of the Editorial Hanging Committee of the British Medical Journal.

1993- : Member of the Data Monitoring Committee of the Medical Research Council Randomised Trial of Hyperthermia in Superficial, Localised Primary and Recurrent Breast Carcinoma.

1996- : Member of the UK Systemic Sclerosis Study Group

Regular Peer Reviewing

British Journal of Ophthalmology, Pharmaceutical Journal, International Journal of Pharmacy Practice.

Occasional Peer Reviewing

British Journal of Cancer, International Journal of Epidemiology, Journal of Clinical Epidemiology, European Journal of Cancer.

Visiting/External Examiner

1980-1983: External Examiner in Medical Statistics, The Queen's University of Belfast.

1983-1986: Visiting Examiner in the Principles of Medical Statistics and Biometry, St George's Hospital Medical School, London.

1983-1984: Visiting Examiner in the Principles of Medical Statistics and Biometry, St Mary's Hospital Medical School, London.

1985-1987: Visiting Examiner in the Principles of Medical Statistics and Biometry, The United Medical Schools of St Thomas' Hospital and Guy's Hospital, London.

1987 External Examiner for a thesis for the degree of M.Sc. in the Queen's University of Belfast (Candidate: Evelyn E. McCrum. Title: Statistical Analysis of Risk Factors Associated with Cardiovascular Disease).

1988-1991: Visiting Examiner in the Principles of Medical Statistics and Biometry, Imperial College of Science, Technology and Medicine, London.

1988-1991: Visiting Examiner in the Principles of Medical Statistics and Biometry, The London Hospital Medical College, London.

1989 Examiner for a thesis for the degree of Ph.D. in the University of London (Candidate: Janet L. Peacock. Title: Birthweight and Cigarette Smoking.

1990-1992: Visiting Examiner in the Principles of Medical Statistics and Biometry University College, London

1992- Examiner in Medical Statistics for the Oncology Fellowship of the Royal College of Radiologists.

1994- Visiting Examiner in the Principles of Medical Statistics and Biometry, Royal Free Hospital School of Medicine, London.

1995- Visiting Examiner in the Principles of Medical Statistics and Biometry, Imperial College of Science, Technology and Medicine, London.

1996- Visiting Examiner in the Principles of Medical Statistics and Biometry, St George's Hospital Medical School, London.

1996- Visiting Examiner in the Principles of Medical Statistics and Biometry, University College, London

PAPERS READ AT CONFERENCES

MacRae KD. An analysis of serial betting behaviour. Experimental Psychology Society, London, January 1967.

MacRae KD, Power RP & Muntz HJ. The use of discriminant analysis in determining the selection of variables for distinguishing among groups. Behavioural Engineering Association, Wexford, September 1971.

Power RP, MacRae KD & Muntz HJ. Discrimination among normals, neurotics and malingerers using the Maudsley Personality Inventory. British Psychological Society, Nottingham, April 1972.

MacRae KD. Statistical considerations in the evaluation of a cooperative study. St Thomas' Hospital Medical School, London, June 1972.

MacRae KD. Progettazione e analisi dell rassegna clinica sul cancro ovarico. Societa Italiana di Ostetricia e Ginecologia, Florence April 1973.

MacRae KD. Further progress in the Ovarian Cancer Survey. St Thomas' Hospital Medical School, London, January 1974.

MacRae KD. The EPI as a clinical instrument. Behavioural Engineering Association, Islay, May 1974.

MacRae KD Statistical aspects of clinical trials for advanced cancer. British Association for Surgical Oncology, London, February 1975.

Muntz HJ, MacRae KD & Power RP Diagnosis by man or machine: a comparison between clinical psychologists and discriminant function analysis. British Psychological Society, Nottingham, April 1975.

MacRae KD Depostat: further progess in the Ovarian Cancer Clinical Survey. South Thames Regional Radiotherapists' Study Day, London, May 1975.

MacRae KD Statistical aspects of trial design. Association of Medical Advisers in the Pharmaceutical Industry, London, May 1976.

MacRae KD The interpretation of pain measurements. Pain Symposium, Guernsey, May 1977.

MacRae KD Clinical trials and tribulations. Northern Ireland Branch of the Royal Statistical Society, Belfast, October 1977.

MacRae KD How are clinical trials of cancer carried out? British Medical Association Symposium, London, November 1977.

MacRae KD Design of the randomised study in early breast cancer. Action Cancer Symposium, Belfast, April 1978.

MacRae KD Clinical trials - statistical principles. Institute of Radiotherapeutics, Glasgow, June 1978.

MacRae KD Statistical aspects of stroke research. International Symposium on Stroke Research, London, June 1978.

MacRae KD Biostatistical consulting. Association of Statisticians in the Pharmaceutical Industry, Stratford-upon-Avon, October 1978.

MacRae KD Evaluation of the results of pain therapy. The Intractable Pain Society, London, October 1978.

MacRae KD Clinical trials in breast cancer: the statistical approach and interpretation of results. British Institute of Radiology, London, October 1978.

MacRae KD Evaluation and validation of subjective measurements. Association for Clinical Research in the Pharmaceutical Industry, London, October 1978.

MacRae KD The epidemiology of breast cancer. Action Cancer Symposium, Belfast, April 1979.

MacRae KD An evaluation of the epidemiological evidence on the risks of oral contraceptives. Schering Workshop on Fertility Control, Berlin, November 1979.

MacRae KD Who needs statistics? Royal College of Radiologists, London, November 1979.

MacRae KD Statistical requirements in clinical trials. Royal Society of Medicine, Section of Measurement in Medicine, London, May 1980.

MacRae KD The general philosophy of clinical trials. Universitat Heidelberg Sonderforschungsbereich, Heidelberg, June 1980.

MacRae KD Health risks of oestrogen therapy. Joint meeting of the Section of Endocrinology of the Royal Society of Medicine, the Society for Endocrinology, and the Medical and Scientific Section of the British Diabetic Association. London, November 1980.

MacRae KD Statistics in family planning research. National Association of Family Planning Doctors, London, October 1981.

MacRae KD The pitfalls of historical controls in clinical trials. Second Heidelberg Symposium on Breast Cancer, Heidelberg, December 1981.

MacRae KD Epidemiology and oral contraception - the way ahead. Workshop on Recent Advances in Oral Contraception, Royal College of Obstetricians and Gynaecologists, London, May 1983.

MacRae KD What epidemiological studies can tell us. Symposium on the Pill in Perspective, London, November 1983.

MacRae KD Statistical problems in clinical trials. Association of Statisticians in the Pharmaceutical Industry, London, June 1984.

MacRae KD Adjuvant therapy for breast cancer. Zentrum fur die methodsiche Betreuung von Therapiestudien bei Brustkrebs, Frankfurt, November 1986.

MacRae KD The value of small trials and large confidence intervals. Third Symposium on Cancer Clinical Trials, Freiburg, February 1987.

MacRae KD What is efficacy: A biostatistical viewpoint. Association for Clinical Research in the Pharmaceutical Industry, Brighton, March 1987.

MacRae KD Epidemiology, encephalopathy and pertussis vaccine. Federation of European Microbiological Societies Symposium, Berlin, April 1988.

MacRae KD Does pertussis vaccine cause brain damage? East Midlands Group of the Royal Statistical Society, Nottingham, June 1988.

MacRae KD Debate - The Place of Chemotherapy in the Treatment of Head and Neck Cancer. Section of Laryngology, The Royal Society of Medicine, Oxford, July 1988.

MacRae KD Does pertussis vaccine cause brain damage? University of London Joint Statistics Seminar (at the L.S.E.) December 1988.

MacRae KD Does pertussis vaccine cause brain damage? Basle Biometric Society, Basle, October 1989.

MacRae KD Overview of major critiques. Workshop on the National Childhood Encephalopathy Study, Institute of Medicine, National Academy of Sciences, Washington DC, November 1989.

MacRae KD A statistician's view of the CRC CEA trial. CRC Gastro-intestinal Trials Annual General Meeting, London, November 1989.

MacRae KD (I) Why is the prospective randomised trial the gold standard? (II) What does a significant result mean? Symposium on Research Methods in Clinical Oncology, Bombay, February 1990.

MacRae KD Should we be at odds with odds ratios? Annual Symposiun of the British Association of Pharmaceutical Physicians, London, June 1990.

MacRae KD Epidemiology and biostatistics in view to family planning. III International Symposium on Contraception, Heidelberg, June 1990.

MacRae KD Statistics in biomedical research. Biomedical Research and Experimental Therapeutics Society of Singapore, Singapore, January 1991.

MacRae KD Misleading statistics. Forum on Clinical Pharmacology and Therapeutics, London, March 1991.

MacRae KD Is a trial worth the trouble? Surgical Study Day, Norfolk and Norwich Institute for Medical Education, Norwich, May 1991.

MacRae KD Current issues in the application of statistics: medicine. Institute of Statisticians One Day Annual Conference, London, May 1991.

MacRae KD How to ensure your next paper is totally unsuitable for publication. European Medical Research Group, London, June 1991.

MacRae KD We can't all be below average. Royal Statistical Society Meeting on Single Population Theories in Epidemiology, London, October, 1991.

MacRae KD The lighter side of statistics. Tenth Anniversary Conference of the Raynaud's and Scleroderma Association, Alsager, September 1992.

MacRae KD Statistics. Workshop for Editors of Journals. British Medical Journal and European Association of Science Editors, Tunbridge Wells, November 1992.

MacRae KD Alcohol is good and bad for you. International Science Festival, Edinburgh, April 1993.

MacRae KD Personal injury - causation. Nottingham Law School Centre of Advanced Litigation 1993 Litigation School, The Law Society, London, October 1993.

MacRae KD Epidemiology and statistics in medicine: what can you believe. Occupational Health Seminar, Royal Society of Medicine, London, June 1994.

MacRae KD Causal inference in pharmacoepidemiology. First International Conference on New Areas of Pharmaceutical Research, Oporto, Portugal, September 1995.

MacRae KD General principles of patient selection and designs. Sixth International Headache Research Seminar. Copenhagen, Denmark, November 1995.

MacRae KD Problems related to phase II and phase III trials: a statistician's view. The 5th European Winter Oncology Conference, Meribel-Mottaret, France, January 1997.

Retsas S, Mohith A, MacRae K, Henry K 1st interim analysis of adjuvant vindesine and DTIC for clinical and histologic involvement of regional lymph nodes in malignant melanoma. 2nd International Conference of the Adjuvant Therapy of Malignant Melanoma. London, March 1997.

Retsas S, Mohith A, MacRae K, Henry K Adjuvant vindesine in malignant melanoma; 20 years on. 2nd International Conference of the Adjuvant Therapy of Malignant Melanoma. London, March 1997.

MacRae KD Effective communication. Royal Statistical Society Quality Forum. London, June 1997.

PUBLICATIONS

MacRae KD (1966) Making diagnosis logical. Zodiac, 13, 1-3.

MacRae KD, & Power RP (1969) Critical comment on one aspect of Graham's "On some aspects of real and apparent visual movement." J Opt Soc Amer, 59, 1002.

MacRae KD (1969) Serial position and sequential dependencies in repeated measures designs. Percept mot Skills, 29, 736-738.

MacRae KD, & Reid JB (1970) Asymmetric stimulus intensity in probability learning. Percept mot Skills, 30, 228.

MacRae KD (1970) Some remarks on Edwards' probability-preference experiments. Percept mot Skills, 30, 300.

Power RP, & MacRae KD (1971) Detectability of items in the Eysenck Personality Inventory. Brit J Psychol, 62, 395-401.

MacRae KD, & Power RP (1972) Real and apparent visual movement. J Opt Soc Amer, 62, 290.

Barr W, Edelstyn GA, Forster DMB, Glennie JMcD, MacRae KD, Menzies DN, O'Sullivan J, Pitchford AG, Smedly GT, Tacchi D, & Ward HWC (1972) Treatment of ovarian carcinoma. Brit med J, 1, 749-750.

Barr W, Edelstyn GA, Forster DMB, Glennie JMcD, MacRae KD, Menzies DN, O'Sullivan J, Pitchford AG, Smedly GT, Tacchi D, & Ward HWC (1972) Treatment of ovarian carcinoma. Lancet, 1, 591.

Edelstyn GA & MacRae KD (1972) Treatment of early breast cancer. Brit med J, 2, 711.

Edelstyn GA & MacRae KD (1972) Treatment of early breast cancer. Brit med J, 3, 587-588.

Edelstyn GA & MacRae KD (1972) Treatment of breast cancer. Lancet, 2, 1307.

MacRae KD & Bullock G (1972) UNI1/UNI2: A Univariate Statistical Package. Pp 18, Queen's University Of Belfast Computer Laboratory.

MacRae KD (1973) Statistical aspects of a co-operative trial on the treatment of ovarian carcinoma. Postgrad med J, 49, 78-80.

Donaldson JD, MacRae KD, Parks TG & Rodgers HW (1973) A multiple discriminant analysis between duodenal ulcer patients and normal controls, using constituents of gastric juice. Europ surg Res, 5 (suppl 2), 8.

Edelstyn GA & MacRae KD (1973) Cyclical combination chemotherapy in advanced breast cancer. Brit J Cancer, 28, 459-461.

Kennedy T, Connell AM, Love AHG, MacRae KD & Spencer EFA (1973) Selective or truncal vagotomy? Brit J Surg, 60, 944-948.

MacRae KD (1973) Progettazione e analisi della rassegna clinica sul cancro ovarico. Communicazione I Relazione Congresso della Societa Italiana di Ostetrica e Ginecologia, Firenze, 522-524.

Donaldson JD, MacRae KD, Parks TG & Rodgers HW (1974) Abnormalities of mucopolysaccharides in duodenal ulceration. Proc roy Soc Med, 67, 11-12.

Power RP, MacRae KD & Muntz HJ (1974) Separation of normals, neurotics and simulating malingerers on the MPI by means of discriminant function analysis. Brit J soc clin Psychol, 13, 65-72.

Donaldson JD, MacRae KD, Parks TG & Rodgers HW (1974) Gas chromatographic measurement of mucopolysaccharides in gastric juice of patients with duodenal ulceration. Gut, 15, 347.

Edelstyn GA & MacRae KD (1975) Breast cancer - mistaken concepts, therapeutic consequences and future implications. J Irish med Assoc, 68, 30-32.

Kennedy T, Johnston GW, MacRae KD & Spencer EFA (1975) Proximal gastric vagotomy: interim results of a randomised controlled trial. Brit med J, 2, 301-303.

Edelstyn GA, Bates TD, Brinkley D, MacRae KD, Spittle MF & Wheeler T (1975) Drugs for common cancers. Brit med J, 2, 502-503.

Edelstyn GA, Bates TD, Brinkley D, MacRae KD, Spittle MF & Wheeler, T (1975) Comparison of 5-day, 1-day, and 2-day cyclical combination chemotherapy in advanced breast cancer. Lancet, 2, 209-211.

Edelstyn GA & MacRae KD (1975) Adriamycin in advanced breast cancer. Lancet, 2, 1095-1096.

Harcus AW & MacRae KD (1975) Progress in the Ovarian Cancer Clnical Survey: a multicentre approach. Chapter 9 in Brush, MG, & Taylor, RW (Editors) Gynaecological Malignancy. Clinical and Experimental Studies. Bailliere Tindall, London, 141-151.

MacRae KD & Power RP (1975) An analysis of the items of the Eysenck Personality Inventory. Brit J Psychol, 66, 501-511.

Gillespie PJ & MacRae KD (1975) Accuracy of liver scintiscanning. J nuclear Med, 16, 1024.

Power RP, Muntz HJ & MacRae KD (1975) Man or machine as diagnostic tool: a comparison between clinical psychologists and discriminant function analysis. Brit J soc clin Psychol, 14, 413-422.

Hood JM, Spencer EFA, MacRae KD & Kennedy T (1975) Value of perioperative acid tests in predicting recurrent ulceration after vagotomy. Brit J Surg, 62, 665.

Edelstyn GA & MacRae KD (1976) Adriamycin in advanced breast cancer. Lancet, 1, 649.

Donaldson JD, MacRae KD & Parks TG (1976) The study of the gastric mucopolysaccharides of patients with duodenal ulceration and X-ray negative dyspepsia by discriminant function analysis. Gut, 17(S), 401-402.

Donaldson JD, MacRae KD & Parks TG (1976) Discriminant function analysis of gastric juice in duodenal ulceration and X-ray negative dyspepsia. Europ surg Res, 8 (Suppl 1), 58.

MacRae KD (1976) Statistical aspects of trial design. Chapter 9 in Good, CS (Editor) The Principles and Practice of Clincial Trials. Churchill Livingstone, Edinburgh, 87-92.

Edelstyn GA & MacRae KD (1976) Early and late breast cancer: a unified concept for treatment. Clin Radiol, 27, 455-462.

Hood JM, Spencer EFA, MacRae KD & Kennedy T (1976) Predictive value of perioperative gastric acid tests. Gut, 17, 998-1000.

Edelstyn GA & MacRae KD (1976) Concomitant androgen therapy in the management of advanced breast cancer by cyclical combined chemotherapy. Clin Oncol, 2, 403-405.

Edelstyn GA, Bates TD, Brinkley D, MacRae KD, Spittle MF & Wheeler T (1977) Short-course cyclical chemotherapy in advanced breast cancer. Lancet, 1, 592.

Edelstyn GA, MacDonald M & MacRae KD (1977) Doxorubicin-induced hair loss and possible modification by scalp cooling. Lancet, 2, 253-254.

Multicentre Cancer Chemotherapy Group (1977) Multimodal therapy for histological stage II breast cancer. Lancet, 2, 396-397.

Power RP & MacRae KD (1977) Characteristics of items of the Eysenck Personality Inventory which affect responses when students simulate. Brit J Psychol, 68, 491-498.

MacRae KD (1977) The interpretation of pain measurements. Chapter 4 in Harcus AW, Smith RB & Whittle BA (Editors) Pain - New Perspectives in Measurement and Management. Churchill Livingstone, Edinburgh, 21-24.

Robinson M, Lonsdale D, MacRae KD & Guz A (1977) The flow-volume curve breathing air or helium-oxygen: an analysis of bias, dispersion and correlation in ten indices and a comparison of non-smokers with asymptomatic smokers. Bull Europ de Physiopath Resp, 13, 96-97.

Edelstyn GA, MacRae KD, Bates TD, Kitchen G, Nicol NT & Spittle MF (1978) Chemotherapy in breast cancer. Lancet, 1, 883.

MacRae KD (1978) One drug for epilepsy. Brit med J, 1, 1215.

Donaldson JD, MacRae KD & Parks TG (1978) Discriminant function analysis of the carbohydrate and nitrogen content of gastric secretion of patients with duodenal ulceration and in control subjects. Irish J Med Sci, 147, 90-96.

MacRae KD (1978) Surgeon-related variables. Lancet, 2, 890.

Edelstyn GA, Bates TD, Brinkley D, Kitchen G, MacRae KD, Nicol NT, Spittle MF & Wheeler T (1978) Multimodal therapy for stage II breast cancer. Lancet, 2, 1092.

Edelstyn GA & MacRae KD (1979) Trials of adjuvant chemotherapy in breast cancer. Lancet, 1, 324.

MacRae KD (1979) Oral hyoscine butylbromide for irritable bowel syndrome? Brit med J, 1, 752.

Doherty JC, MacRae KD & Platt NE (1979) Treatment of chronic eczemas: the comparative efficacy of two creams. The Practitioner, 222, 561-563.

Edelstyn GA, MacRae KD & MacDonald FM (1979) Improvement in life quality in cancer patients undergoing chemotherapy. Clin Oncol, 5, 43-49.

MacRae KD (1979) Statistical aspects of clinical trials. Chapter 21 in Greenhalgh RM & Clifford Rose F (Editors) Progress in Stroke Research 1, Pitman Medical, Tunbridge Wells, 176-180.

Edelstyn GA, Jeffrey L & MacRae KD (1979) Nolvadex (tamoxifen) following combination chemotherapy in progressive advanced breast cancer. Clin Oncol, 5, 325-330.

Spittle MF, Bates TD, Kitchen G, Nicol NT, Wheeler T, Edelstyn GA & MacRae KD (1980) Combination chemotherapy in advanced breast cancer. Clin Oncol, 6, 153-157.

Eiser NM, Mills J, MacRae KD, Snashall PD & Guz A (1980) Histamine receptors in normal human bronchi. Clin Sci, 58, 537-544.

MacRae KD (1980) An evaluation of the epidemiological evidence on the risks of oral contraceptives. Roy Soc Med Internat Cong & Symp Series, 31, 13-20.

McIvor J, Massouh H, Backhouse BM & MacRae KD (1980) Lymphography in prostatic carcinoma - implications for the diagnosis of metastases. Brit J Radiol, 53, 74-80.

MacRae KD (1980) Thrombosis and oral contraception. Brit J Hosp Med, 24, 438-442.

MacRae KD (1981) Thrombosis and oral contraception. Brit J hosp Med, 25, 205-206.

MacRae KD (1981) Thrombosis and oral contraception. Brit J hosp Med, 25, 421.

Wiseman RA & MacRae KD (1981) Oral contraceptives and the decline in mortality from circulatory disease. Fertil & Steril, 35, 277-283.

Donaldson JD, MacRae KD & Parks TG (1981) The assessment of mucus substances in gastric juice from duodenal ulcer patients and normal subjects. Scand J Gastroent, 16, 235-239.

Wheeler TK, Edelstyn GA, Bates T, Brinkley D, Evans RGB, Kitchen G, MacRae KD, Nicol NT & Spittle MF (1979) Four-drug combination chemotherapy following surgery for breast cancer. In Jones SE & Salmon SE (Editors) Adjuvant Therapy of Cancer II, Grune & Stratton, New York, 269-276.

MacRae KD (1981) Health risks of oestrogen therapy. J Endocr, 89, 145-148.

Eiser NM, MacRae KD & Guz A (1981) Evaluation and expression of bronchial provocation tests. Bull Europ de Physiopath Resp, 17 427-440.

MacRae KD (1981) Thrombosis and oral contraception. Brit J hosp Med, 26, 185-186.

Wiseman RA & MacRae KD (1981) Oral contraceptives and circulatory disease. Fertil & Steril, 36, 414-416.

Law RG & MacRae KD (1982) Head circumference as an index of fetal age. J ultrasound Med, 1, 281-288.

MacRae KD (1982) The pitfalls of historical controls in clinical trials. In Baum M, Kay R & Scheurlen H (Editors) Clinical Trials in Early Breast Cancer. Birkhauser Verlag, Basel, 312-315.

MacRae KD & Wright JT (1982) Analgesia and satisfaction in childbirth. Lancet, 2, 992.

Donaldson JD, MacRae KD & Parks TG (1983) Comparison of mucus substances in gastric juice of normal subjects, duodenal ulcer, and dyspeptic patients. Europ surg Res, 15, 11-17.

Wright JT, Waterson EJ, Barrison IG, Toplis PJ, Lewis IG, Gordon MG, MacRae KD, Morris NF & Murray-Lyon IM (1983) Alcohol consumption, pregnancy, and low birthweight. Lancet, 8326, 663-665.

MacDonald AJR, MacRae KD, Master BR, & Rubin AP (1983) Superficial acupuncture in the relief of chronic low back pain. Ann roy Coll Surg of England, 65: 44-46.

Price LA, MacRae KD & Hill BT (1983) Integration of safe initial combination chemotherapy (without Cisplatin) with a high response rate and local therapy for untreated stage III and IV epidermoid cancer of the head and neck: 5-year survival data. Cancer Treat Rep, 67, 535-539.

Van Riel PLCM, van de Putte LBA, Gribnau FWJ, MacRae KD & de Rooy DJAM (1983) A single-blind comparative study of auranofin and gold thioglucose in patients with rheumatoid arthritis. In Capell HA, Cole DS, Manghani KK & Morris RW (Editors), Auranofin. Excerpta Medica, Amsterdam, 135-145.

McCollum C & MacRae KD (1983) Prevention of drip phlebitis by inline filtration. Brit J Surg, 70, 449.

MacRae KD & Wright JT (1983) The controlled clinical trial and the advance of reliable knowledge. Brit med J, 287, 1217-1218.

Wright JT, MacRae KD, Barrison IG & Waterson EJ (1984) Effects of moderate alcohol consumption and smoking on fetal outcome. In Porter R & O'Connor M (Editors) Mechanisms of Alcohol Damage in Utero (CIBA Foundation Symposium 105). Pitman, London, 240-253.

MacRae KD (1984) What epidemiological studies can tell us. J Obstet & Gyn, 4 (Suppl 2), S79-S87.

Baum M & MacRae KD (1984) Screening for breast cancer. Lancet, 2, 462.

Van Riel PLCM, van de Putte LBA, Gribnau FWJ & MacRae KD (1984) Comparison of auranofin and aurothioglucose in the treatment of rheumatoid arthritis: a single blind study. Clinical rheumatology, 3: (Suppl 1), 51-56.

Seddon L, Wright JT & MacRae KD (1985) Determinants of breast feeding in primigravidae. J Obstet & Gyn, 5, 165-166.

MacGregor GA, Sagnella GA & MacRae KD (1985) Misleading paper about misleading statistics. Lancet, 1, 926-927.

Guz A, MacRae K, Murphy K, Shea S & Walter J (1985) The effect of opening the eyes and reading on resting ventilation in man. J Physiology 365, 101P.

MacRae K (1985) Exaggerated responsiveness to thyrotrophin releasing hormone. Brit med J 291, 485-486.

Price LA, Hill BT & MacRae KD (1985) Increased survival in stage III and IV squamous cell carcinoma of the head and neck using an initial 24-hour chemotherapy protocol without cisplatin: 6 year follow-up. Primary Chemotherapy in Cancer Medicine, 159-167. Alan R Liss Inc.

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The Multicentre Trial Group (EGL Bywaters, AV Camp, AT Day, JR Golding AM Freeman, DN Golding, CL Greenbury, AGS Hill, HFH Hill, M Webley, IM Koch, WH Lyle, KD MacRae, AJ Swannell) (1986) A prospective five-year comparison of treatment which included penicillamine with that excluding pernicillamine in early

rheumatoid arthritis. Brit J Rheumatol, 25, 184-192.

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October 6, 1997

Dear Sir

Re: Federal Register, Vol 62, No 13, p3727: NTP Report on Carcinogens. Ninth Edition

We understand that oral use of smokeless tobacco products is proposed for listing in the Ninth Edition of the NTP Report on Carcinogens. We have conducted an extensive review of the scientific literature on smokeless tobacco in order to examine the experimental evidence relating to smokeless tobacco and oral cancer. Overall, the experimental studies support the conclusion that smokeless tobacco is not carcinogenic in the oral cavity of laboratory animals. Moreover, it is our judgement that those experimental studies do not support the listing of oral use of smokeless tobacco products in the NTP's Ninth Report on Carcinogens.

We have been requested by United States Tobacco Company, which funded our work, to provide you with the enclosed copy of our monograph entitled "Assessment of the Experimental Evidence Relating to Smokeless Tobacco and Oral Cancer", issued on October 31, 1996. The monograph describes the experimental studies in detail and the justification for our conclusions. Since preparing this monograph, we have continued to scan the relevant literature and have seen nothing which would lead us to modify our conclusions.

Yours faithfully,

Professor Paul Grasso, MD, FRCPath

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Assessment of the experimental evidence relating to smokeless tobacco and oral cancer

Report No. RI93/TOX/001

This work was funded by the United States Tobacco Company

Issued by: Professor P Grasso, Dr D Benford and Mr A H Mann
Date issued: 31st October 1996

Head of Institute
Professor D A Stubbs

World Health Organization Collaborating
Centre for the Protection of Drinking Water
Quality and Human Health

Health for all by the year 2000

EXECUTIVE SUMMARY

The purpose of this monograph is to review experimental data dealing with smokeless tobacco as a possible cause of oral cancer. This review has been undertaken mainly because anecdotal reports and epidemiological studies have raised a question about the relationship between the use of smokeless tobacco and oral cancer in humans. However, the anecdotal reports lack scientific underpinning while the epidemiological studies are confounded by a number of other factors which are associated with oral cancer. These include poor oral hygiene, socio-economic status, diet and alcohol.

Long-term studies in experimental animals of different species indicate that smokeless tobacco is not carcinogenic in the oral cavity. When the epithelium of the hamster cheek pouch was exposed to large quantities of smokeless tobacco for long periods there was no resulting oral cancer although parallel studies showed that the cheek pouch epithelium was sensitive to known chemical carcinogens at much lower levels of exposure. In studies in which smokeless tobacco was inserted into a surgically- constructed lip canal in the rat there was an incidence of tumours of the connective tissue and the epithelium of adjacent structures. These tumours can be explained however by the mechanical trauma of the procedures used.

Chemical analysis of smokeless tobacco indicates the presence of a class of compounds called tobacco specific nitrosamines (TSNAs). Some of these compounds, when tested in pure form, ie isolated from smokeless tobacco, cause both benign and malignant tumours in some organs of laboratory rodents. These TSNAs, however, do not cause cancer in the oral cavity of laboratory animals. Their carcinogenic effect in the other organs is considerably reduced if the experimental animals are treated with certain plant products, including tobacco itself, just prior to or at the same time as exposure to the tobacco-specific nitrosamines (TSNAs).

Examination of the mechanisms by which TSNAs are metabolised in mammals has confirmed that, like other carcinogenic nitrosamines found in food and other plant materials, they form metabolites which are capable of interacting with cellular genetic material such as DNA. However, the production of these agents in the different organs of experimental animals does not correlate very well with the sites of tumour formation. Some of the plant products such as alkylarylisothiocyanates, which are commonly found in cruciform vegetables have been shown

to reduce the carcinogenic potential of TSNAs in long-term animal studies and they also inhibit the metabolism of TSNAs to agents which interact with DNA.

For many years mutagenicity studies have been used as indicators of potential carcinogenicity. Smokeless tobacco itself, because of its physical nature, cannot easily be tested in experimental mutagenicity procedures but the TSNAs and extracts of smokeless tobacco have been shown to be mutagenic. The observation of mutagenicity is supported by the demonstration that metabolic products from TSNAs interact with DNA. In the absence of results from long-term animal studies these mutagenicity results would have been interpreted as indications that smokeless tobacco was potentially carcinogenic. However, the negative results in the animal carcinogenicity studies are better indicators of the potential carcinogenicity of smokeless tobacco than the results of the mutagenicity tests.

Some viruses, particularly the herpes simplex virus, are associated with the incidence of cancer. It has been shown experimentally that there was a high incidence of oral cancer in hamsters when snuff was applied repeatedly to the oral cavity if an active infection with herpes simplex virus was artificially maintained for the whole of the experimental period. No tumours were observed in hamsters or rats treated similarly with smokeless tobacco when the virus infection had been allowed to become dormant.

Overall the experimental studies support the conclusion that smokeless tobacco has not been shown to be carcinogenic in the oral cavity of laboratory animals.

CONTENTS

CONTENTS (continued)

This report is the result of a survey of literature up to the end of 1992 and includes some selected references from 1993.

ACKNOWLEDGEMENTS

We should like to express our thanks to Dr A D Furst and Dr T Malmfors for their helpful comments on our text.

1 HISTORICAL BACKGROUND

1.1 Introduction

The tobacco plant (Nicotiana tabacum and N rustica) is thought to have originated on the mainland between North and South America over 7000 years ago and its cultivation is of considerable antiquity (Voges, 1984). According to Christen *et al* (1982), American Indians were the first to smoke, chew and snuff tobacco. The origins of these habits are lost in antiquity but some clues suggest that they may have afforded some relief from the rigours of life in those ancient times as well as being a pleasurable pastime. Thus, according to Heimann (1960) and Stewart (1967) the explorer Amerigo Vespucci thought that the inhabitants of a small island off the coast of Venezuela chewed tobacco to quench thirst because the island was very short of water and chewing tobacco induced profuse salivation. Chewing tobacco was also found to assuage the effect of fatigue and hunger as well as thirst; in fact, it has been reported that an Indian could trek for 2 or 3 days with no other support than tobacco. Tobacco was also thought to have medicinal properties by the Indians and to be a good cleansing agent for teeth. No doubt, the fibrous nature of the tobacco may have been an effective tooth cleanser in the absence of any other more suitable material.

Tobacco was introduced into Europe soon after the discovery of the American continent and by the beginning of the 17th century it arrived in Turkey, Russia, Arabia and the Far East (Axton, 1975).

1.2 Chewing tobacco

According to Gottsegen (1940) and Brooks (1952) tobacco chewing became popular in Eastern USA during the first half of the 19th century and the custom spread to Europe. Tobacco chewing was found to be a good substitute for smoking by those who could not smoke or who were prevented from doing so, for example, sailors chewed tobacco because of a ban on smoking which constituted a fire hazard on board (Brooks, 1952). The belief in the beneficial effect of tobacco chewing against illness was also prevalent in Europe and no doubt helped to popularise the product.

There was an undesirable side to the practise of chewing tobacco. Some users expectorated the chewed cud indiscriminately, thus creating a nuisance in public places while the advent of the germ theory of disease led to the fear that such practices may present a serious hazard to health by disseminating germs, particularly tuberculosis (IARC, 1985). Thus the practice of chewing tobacco became less popular towards the end of the 19th century and the beginning of the 20th century but is still widespread today.

1.3 Inhaled powdered tobacco

The practice of inhaling powdered tobacco is thought to have originated among the Indians of Brazil. They ground the tobacco leaves to a powder in a rosewood cup and inhaled the powdered leaves via ornate bone tubes (Curtis, 1935). According to Christen *et al* (1982) and Stewart (1967) the practice of inhaling powdered tobacco leaves was widespread among the inhabitants of Mexico and the Caribbean Islands, notably in Haiti and the lesser Antilles. Snuff was introduced to Europe by members of the second expedition of Columbus to the New World and at first it was thought to possess medicinal properties. The inhalation of snuff gradually spread throughout the then known world and became a socially acceptable practice particularly in Europe. Snuff use reached a peak in England during the reign of Queen Anne (1702-1714). It continued to be popular for several decades afterwards and, according to records, it was used by several prominent people such as Lord Nelson, the Duke of Wellington, Alexander Pope and Samuel Johnson.

1.4 A note on current practices

Chewing tobacco and snuff are currently called smokeless tobacco. Snuff consists of tobacco that has been cured and then finely ground to produce dry (Scotch) snuff (less than 10% moisture), moist snuff (up to 50% moisture), or fine-cut tobacco, the latter being generally considered a form of moist snuff.

The customary use of snuff involves "snuff dipping". It consists of taking a small amount of snuff between the gingiva and either the lip or the buccal mucosa. It then either can be left for a few minutes and cleared by expectoration or left for much longer periods; some users retain snuff in this position for many hours. Snuff dippers usually expectorate saliva mixed with tobacco extract.

Chewing tobacco can be obtained as plug, loose-leaf and twist or roll tobacco. Plug tobacco is made from tobacco leaves which are wrapped in fine tobacco and pressed into flat bars or rolls. Loose-leaf tobacco is formed from fermented cigar leaf tobacco which is not compressed while twist or roll tobacco is composed of cured, flavoured leaves which have been twisted into strands and dried. Chewing tobacco is held in the mouth where it can be chewed intermittently for several hours. The saliva mixed with tobacco extract is usually expectorated.

The use of smokeless tobacco varies considerably from state to state in the USA and from country to country in Western Europe and the Far East (Hunter *et al*, 1986; Marty *et al*, 1986). In the USA the

custom of using smokeless tobacco is particularly prevalent among the native Indian population (Cullen *et al*, 1986). The consumption of smokeless tobacco in the United States is reported to have tripled between 1972 and 1991 when it is estimated to have involved 5.3 million adults (Anon, 1993). These smokeless tobacco users were predominantly men and represented 5.6% of the adult male population. The greatest usage was in the native American and non-Hispanic white males and the usage was inversely correlated with the number of years in education. In Sweden about 15% of adult males use moist snuff (Lewin *et al*, 1994).

Recently chewing tobacco has been offered to users enclosed in fabric material of the same type as that used in making tea bags. About 1 gram of tobacco is contained in a bag approximately 2" square. The bag is placed between the gingiva and the cheek and is either left *in situ* or chewed intermittently. This method of presenting snuff tobacco allows extraction of nicotine by saliva and improves oral hygiene in tobacco chewers by retaining the fibrous residue in the bag.

Over the years there have been many articles in the scientific literature dealing with smokeless tobacco and oral cancer. A number of epidemiological studies have been conducted to ascertain whether smokeless tobacco is associated statistically with this disease (IARC, 1985). The results of the epidemiological studies are inconclusive. In addition, a large amount of experimental work has been carried out to investigate the possible carcinogenicity of smokeless tobacco. Various models have been employed in this endeavour. The purpose of the current monograph is to review the results obtained in experimental research with the smokeless tobacco products used in USA and Western Europe. The monograph does not deal with cigarette tobacco or the smokeless tobacco products used in Asia because these are substantially different from US or Western European smokeless tobacco. The Asian smokeless tobacco products frequently incorporate materials such as slaked lime and betel nut.

2 LABORATORY ANIMAL STUDIES ON WHOLE SMOKELESS TOBACCO OR TOBACCO EXTRACTS

2.1 The hamster cheek pouch

The hamster possesses two pouches, one on either side of the mouth, which open into the oral cavity and lie underneath the muscles of the cheek hence the name "cheek pouches". The openings of the pouches lie in the anterior part of the oral cavity and are associated with small salivary glands which produce both serous and mucous secretions. The pouches extend backwards along the oral cavity but do not reach as far as the oropharyngeal junction.

Histologically, the epithelium of the cheek pouch is stratified squamous. It is 2 to 4 cells thick and consists of a well-defined basal layer (but no rete pegs), a spinous layer, some granular cells and a slightly keratinised stratum corneum. The connective tissue layer underneath the epithelium (lamina propria) contains no accessory structures but may contain unusually large fat cells. A layer of longitudinally arranged skeletal muscle fibres is found underneath the "lamina propria" and outside the muscle there is a layer of loosely packed areolar tissue. These two layers form the submucosa which in turn is covered by dense subcutaneous tissue and skin. Basically, the cheek pouch is surrounded by skeletal muscle and loose areolar tissue. When the pouch is separated from the surrounding tissue, the line of separation occurs at the layer of loose areolar tissue.

Functionally, the cheek pouch stores half-chewed food which is pressed out as needed. Tobacco and snuff, when introduced into the pouch, remain in situ for several hours and small amounts are periodically extruded and chewed (Shklar *et al*, 1985). Thus, the hamster cheek pouch facilitates the study of prolonged exposure of the oral mucosa to tobacco products.

One of the earliest studies of the possible carcinogenicity of smokeless tobacco was carried out in this model by Peacock *et al* (1959, 1960). The authors questioned the relevance of the tumours obtained in earlier studies involving repeated paintings of distillates of cigarette tobacco on mouse skin for assessing the carcinogenicity of unburned tobacco. They therefore selected the hamsters' pouch for conducting their study. In the authors' experience, absorption of compounds from the pouch into the systemic circulation was slow. For example, implantation of 40mg strychnine (8 times the minimum lethal dose for hamsters) into the pouch did not result in the rapid deaths which would have occurred from direct oral dosing and some of the animals survived indefinitely. The slow absorption meant that prolonged contact was possible between the material inserted and the epithelium of the pouch.

In their experiments Peacock *et al*, (1959 and 1960) used 124 hamsters (sex unspecified). After dissecting out the cheek pouch and widening the oral opening of the pouch, the authors inserted $10cm^3$ of snuff or $2cm^3$ of chewing tobacco plug and then ligated the sac and returned it to its original position underneath the cheek muscle. Sixty (60) hamsters received the snuff and the other 64 received chewing tobacco. The test material was implanted in the left pouch while the materials used as controls (sand or some bland material) were implanted in the right pouch. Refilling the cheek pouches with smokeless tobacco was attempted in a few instances but this resulted in leakage into the surrounding tissues and subsequent death of the animals. A mild chronic infection, which appeared to be self-limited, occurred in a few of the pouches and did not progress beyond 3 weeks. The experiment lasted 30 months. In the

group that received snuff, 39 died within the first 12 months, 11 within the next 12 months and the 10 survivors within the next six months. In the group that received chewing tobacco, 43 had died within the first 12 months, 13 within the next 12 months and the remaining 8 within the next 6 months. No tumours were found in the mucous membrane of the pouch or oral cavity in any of the groups.

Dunham and Herold (1962) investigated the possible carcinogenicity of snuff in a group of 35 hamsters. The snuff was incorporated into a beeswax pellet and inserted through an incision into the isolated cheek pouch. After insertion a 4-5 mm loop of steel wire was made around the neck of the pouch. The pellet contained 20% of snuff and was left in place until the end of the experiment at 2 years. An inflammatory lesion of the pouch was observed in only two animals. No tumours were observed in the pouch or oral cavity. The laboratory carcinogens 7,12-dimethylbenzanthracene (DMBA) and 3-methylcholanthrene (MCA) were incorporated into beeswax in the same way as snuff and inserted into the cheek pouch of 71 hamsters and served as positive controls. Serial killings in this group during the first five months showed the development of acute inflammation, ulceration and necrosis of the mucus membrane of the cheek pouch. Several carcinomas and sarcomas developed in the positive controls after 6 months.

In a later experiment by Dunham, Muir and Hamner (1966) snuff was tested in a group of 7 hamsters. At the termination of the experiment the average age was 99 weeks. Fifty (50) mg of snuff were inserted by a child size nasal speculum once daily for 5 consecutive days each week throughout the experiment except in the first two weeks when the amount administered was 250mg/insertion. Starch powder was used as a negative control. There were no reactive changes or tumours in the pouches of hamsters treated with snuff or with starch powder. In another experiment in which Dunham was also a principal author, chow containing 2.5% of snuff was offered to a group of 2 male and 2 female hamsters daily for 5 days a week for up to 2 years. No tumours were observed (Dunham, Snell and Stewart, 1975).

Homburger (1971) investigated the possible carcinogenic properties of smokeless tobacco by using the oral mucosa as well as the cheek pouch of hamsters. He immobilised the animals' heads by a stanchion for 30 minutes each day, which allowed snuff to be applied with an automatic cartridge filler to the gingivolingual area, including the upper part of the buccal pouch. One experiment was terminated at 8 months according to the author's table (30 weeks according to the text) because of high mortality. In another experiment groups of 35 males and 25 females were allocated to one of the following treatments: snuff, cotton (dry, as control), benzo(a)pyrene (BaP) and DMBA. The snuff was applied neat, while the carcinogens were applied on absorbent cotton as 0.2ml of a 0.5% solution in acetone or peanut oil. Daily exposure continued for one year except for DMBA which was carried out only for 30 weeks because animals' health deteriorated. The experiment was terminated after one year.

One male and two females were lost from untreated controls. In contrast, the numbers of survivors for males and females respectively in the group treated with dry cotton were 15 and 14, in the snuff treated group 15 and 9, in the BaP treated group 17 and 7, in the DMBA group 10 and 4.

From the beginning of the experiment, hamsters exposed to snuff were noted to cease to struggle and some actually went to sleep presumably because of the calming effect of the tobacco. In contrast, animals exposed to cotton or to carcinogens continued to struggle during the 30 minutes of restraint. The authors attributed the high mortality to cervical dislocation brought about by the struggle against restraint.

The epithelium of the lip of the oral cavity and of the cheek pouch of the animals exposed to snuff showed only minor changes compared with controls. Focal epidermal hyperplasia (i.e. an increase in cells) was observed microscopically in 6 snuff-exposed and 2 cotton control animals (sex unspecified). One benign tumour (papilloma) was found in each of the snuff-exposed and cotton control groups.

A marked hyperplasia and metaplasia (change in cell morphology) occurred in the majority of animals treated with BaP. Similar but less severe lesions occurred in hamsters treated with DMBA. The difference in severity of the lesions induced by DMBA is probably due to the shorter duration of treatment. Nevertheless, 10 squamous cell carcinomas, 3 in the pouch, 5 in the skin and 2 in the mouth, developed in the DMBA exposed group whereas only 3 tumours were observed in the group of animals treated with BaP, one each in skin, oral and pouch mucosa.

In another short-term study with male hamsters, 70mg of finely powdered snuff or 50 to 100mg of coarser tobacco were introduced daily for 20 weeks into the cheek pouch of groups of 20 animals. The experiment was terminated at 20 weeks. While no significant pathological changes were observed in these animals there was a slight diminution of mitotic activity and an increase in Langerhan's cells (Shklar *et al*, 1985).

Similarly the daily application of 2g of commercially available American snuff to the blind end of the right buccal pouch of a group of 8 male hamsters daily, 5 days a week for 6 months (terminated at 6 months) resulted in hyperplasia (increase in cells) of the buccal cavity epithelium. A roughening of the surface was observed by scanning electron microscopy while visually whitish patches were observed (Worawongvasu, *et al*, 1991). No focal proliferative lesions or tumours were found.

Comment

The investigation carried out by Peacock *et al* (1959-1960) and Dunham *et al* (1966) on the hamster cheek pouch provide substantial evidence that smokeless tobacco is not carcinogenic. Although there was a high mortality in the early months of both experiments, some animals survived beyond 24 and up to 30 months in both studies. Despite the relatively small number of these survivors, the duration of exposure was prolonged enough to allow detection of a carcinogenic effect, even a weak one. Observations from short-term studies support this view. Thus the daily insertion of snuff for about 5-6 months produced only a mild hyperplasia (increase in cell number) without signs of early tumour development such as dysplasia (abnormal cell disposition in an epithelium) or focal proliferative lesions (Shklar *et al*, 1985; Worawongvasu *et al*, 1991).

Furthermore, the development of tumours in the cheek pouch by the classical polycyclic aromatic hydrocarbon carcinogens, BaP, MCA and DMBA, after a latent period of only a few months (Dunham and Herold, 1962; Homburger *et al*, 1971) clearly indicates that the cheek pouch is sensitive to carcinogens. The absence of the production of any tumours (including benign) or of lesions considered to herald the production of neoplasia by snuff is in strong contrast with the action of carcinogens and supports the view that snuff does not possess any carcinogenic properties. Furthermore no increase in systemic tumour incidence was reported in any of the studies.

2.2 Topical application

The identification of certain carcinogenic tobacco-specific nitrosamines (TSNAs) in smokeless tobacco raised questions about smokeless tobacco. Hecht, Rivenson *et al* (1986) devised a protocol in which groups of rats were treated by application to the oral cavity of either aqueous extracts of snuff, aqueous extracts of snuff to which 10 times the innate concentrations of N- nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) had been added, or aqueous solutions of NNN and NNK equal to the amount added to snuff extract (see also Sections 3.2 and 3.3 of this monograph). 0.5ml of each of these solutions was absorbed onto a cotton swab and painted over the oral cavity and lips of rats until the entire amount in the swab has been used. This procedure was carried out once a day for the first seven days. From weeks 2-23 it was carried out once daily on Tuesday and Thursdays and twice a day on the other days of the week and twice daily from week 24 to 131. The experiment was terminated at 131 weeks. No tumours were observed in the group of 30 rats treated with snuff extract. NNN and NNK produced a statistically significant increase in benign tumours of the oral mucosa. Snuff extract enriched with the same amount of NNN and NNK, however, produced a lower incidence of these

benign tumours (3 v 9, see Table 4) suggesting that snuff, or some component of snuff, may have an inhibitory effect on the tumorigenicity of nitrosamines.

In a study on twelve rhesus monkeys (Smith, Mincer *et al*, 1970) the oral mucosa was exposed to snuff by various devices for periods of 3 to 7 years. No neoplasms were found after these treatments.

Comment

When snuff or snuff extract was topically applied to the oral mucosa of laboratory rats, results did not indicate a carcinogenic response. The presence of snuff extract reduced the incidence of benign tumours caused by the topical application of NNN or NNK suggesting an inhibiting effect of the snuff or some component of it.

2.3 Dietary administration

DiPaulo (1962) fed male Wistar rats diets containing dried snuff for 18 months and male mice (DBA or C57 B1 strain) for 15 months. There were 40 rats, 34 DBA mice and 16 C57B1 mice in the treated groups at the beginning of the study. The dietary concentrations of dried snuff were approximately 5% for the rats and 25% for the mice at the beginning of the study falling by stages to 5% at the end. The snuff-treated groups had statistically significantly reduced body weight and survival compared with the controls. Histopathology was carried out on tissues which appeared grossly abnormal and this showed "few pathological changes". In the snuff-treated groups one rat had a kidney sarcoma and there were leukaemias in one rat and in three DBA mice. No malignancies were reported in the controls although the authors comment that tumours occur spontaneously in these animals. No malignancies of the oral cavity or upper alimentary tract were found in the snuff-treated test groups.

Homburger *et al* (1976) having previously obtained negative results using the hamster cheek pouch (see Section 2.1) turned to the dietary route for further investigation. According to the authors' text 500 male hamsters were employed in this study, 250 of the BIO 15.16 strain and 250 of the BIO 87.20 strain. However, according to the table of neoplasms in the paper both male and female hamsters were used. The hamsters of each strain were divided into 5 groups. The first and second groups were given a diet containing 20% snuff, or 20% cellulose; the third group received 50 doses of 5mg methylcholanthrene (MCA) by gavage (frequency not stated). The fourth and fifth group received a low dose of MCA (0.5mg x 50 doses) by gavage, as well as a diet containing either 20% MCA or 20% snuff.

Food consumption was reduced in all animals receiving the diet containing snuff but the difference was not statistically significant. The body weight of hamsters of the BIO 87.20 strain maintained on 20% snuff was significantly less than for those on the same regimen plus MCA by gavage. The bodyweight of the BIO 15.16 hamsters dosed with snuff was only slightly reduced.

The experiment continued for 2 years at which time it was terminated. Twenty-two out of the total of 100 animals treated with high dose of MCA had died during the first year, but only 10 of all remaining animals died during this period. Cotinine was detected in the serum of animals receiving snuff.

No increase in tumour incidence was observed in animals fed snuff compared with those fed cellulose, indicating that the commercial brand of snuff used in this study was not carcinogenic (Table 5). Forestomach tumours were observed in thirteen of the hamsters receiving MCA and cellulose and seven of these were malignant. Three forestomach tumours of which two were malignant, were observed in hamsters treated with MCA and snuff.

Comment

Dietary studies with snuff clearly indicate that it is not carcinogenic.

The study by Hornburger *et al* (1976) is valuable because it lasted for approximately two years and was carried out on adequate groups of animals. Furthermore the concentration of snuff was 20% of the diet - a proportion much higher than that normally used for non toxic material (5%) - suggesting that the dose level was sufficiently high to reveal any carcinogenic potential.

The inclusion of cellulose as a "negative" and of MCA as a "positive" control further adds to the importance of this study. MCA is clearly carcinogenic producing tumours in the forestomach, the glandular stomach and intestine. In strong contrast there were a few tumours in the forestomach only in groups dosed with snuff and cellulose. The forestomach is an organ which is known to be prone to develop tumours in rodents if subjected to repeated trauma. There is an indication that snuff might have an inhibiting effect on tumour production by MCA since the number of tumours induced by snuff and MCA is smaller than those induced by cellulose and MCA.

2.4 Artificial lip canal in the rat

Hirsch and Thilander (1981) developed a rat model that sought to simulate human "dipping" by facilitating contact between snuff and saliva. Using microsurgical techniques, they created an artificial canal in the lower lip of a young adult rat which was open at both ends and which was lined internally by mucosal epithelium and externally by skin from the lip. Tobacco or similar solid products could then be inserted and replaced readily as required by the experimental protocol. The authors do not state the interval of time between the operation and the first insertion into the lip canal. The operation initially caused a marked inflammatory reaction but in one animal, killed 14 days later the reaction had almost completely subsided. At this time the lumen of the canal was covered by keratinised squamous epithelium which was acanthotic (5-10 cell layers) with rete pegs projecting into the submucous layer.

As far as one could ascertain, the tissue reaction at the site of surgery was allowed to heal before being used for experimental purposes. The test material was injected from the lip side into the artificial canal by a plastic syringe until excess snuff was pressed out through the buccal opening, ensuring complete filling of the canal. The authors found that this model could accommodate approximately 0.29g of powdered snuff which corresponds to a mean dose of 1g/kg body weight (approximately 5 times the amount a human might use). The inserted material was retained for 5 to 8 hours and was accompanied by "hypersalivation" and an increase of blood nicotine from 13ng/ml (in one control) to 83 and 250 ng/ml (two animals). After a twice-daily application of 0.2 to 0.4 g of powdered snuff for 9 months the epithelium of the canal was mildly to moderately hyperplastic and the adjacent connective tissue exhibited an inflammatory reaction which varied in degree from mild to severe (Hirsch and Thilander, 1981). This model was utilised by Hirsch and Johansson (1983) to study the effect of long-term application of snuff on the oral mucosa of the rat. These authors inserted 0.2g of standard snuff or snuff made more alkaline than normal by the addition of sodium carbonate into an artificially created lip canal twice daily for 9 to 22 months. Out of 42 male and female rats that received the standard snuff, groups of 6 to 8 of each sex were killed at 9 and 12 months. The rats remained healthy up to 18 months, after which there was a decline so that the remainder were killed between 18 and 22 months when moribund. The 10 rats that received the alkaline snuff were killed when moribund between 18 and 22 months. The 15 rats in the control group (which had the lip canal but were not treated with snuff or any other material) were killed in 3 groups at 9, 12 and 18 months.

The squamous epithelium of the canal exhibited a generalised mild to moderate hyperplasia after 9 to 12 months of treatment with standard snuff, but foci of severe hyperplasia also occurred. A disturbed

polarity of epithelial cells was observed in some of the foci. The underlying connective tissue exhibited a mild to moderate inflammatory reaction at 9 months.

The reactive lesions in the epithelium and submucosa showed virtually no further changes as the experiment progressed but the fibrosis which was observed in the submucosa at 9 months became more prominent later on. The histological picture produced by alkaline snuff in the tissues of the lip canal was similar to that produced by standard snuff while only a minimal to mild epithelial hyperplasia was observed in untreated controls. A single invasive squamous cell carcinoma was found in the oral cavity of a rat which had been treated with standard snuff for 8½ months which was considered to be spontaneous or induced by treatment. The exact site of the carcinoma within the oral cavity was not specified by the authors. Rats which had been exposed to snuff for 10 to 22 months had a marked papillary hyperplasia of the forestomach, an organ not found in humans (Hirsch and Johansson, 1983).

The appearance of some dysplastic foci in the hyperplastic epithelium of the lip canal in animals repeatedly treated with snuff prompted a follow-up investigation to find out whether these dysplastic foci would be followed by the development of tumours if treatment ceased (Hirsch, Larsson and Johansson, 1986). 0.2g of commercially available snuff were inserted twice daily into the artificial lip canal of 30 male Sprague Dawley rats for 13 months. Ten rats were killed at the end of the treatment period, another 10 were killed one month later and the last group were killed 4 months after cessation of treatment. Ten control rats were subjected to the same surgical procedure but were left untreated and were killed at the 13th month of the study. Histologically, a slight hyperkeratosis and acanthosis was present in the mucosal epithelium of the lip canal in this group but the rete pegs were not prominent and the inflammatory reaction in the subepithelial tissue was mild or absent. In the test animals killed at the same time there was a mild to moderate squamous hyperplasia and hyperkeratosis. Acanthosis was slight or moderate with marked development of rete pegs and focal atypia in the basal layer. The inflammatory reaction in the submucous connective tissue varied from slight to severe but fibrosis was prominent and severe in all animals. The reaction of the mucosal epithelium and the inflammatory infiltrate were less prominent after 1 or 4 months' treatment-free period but the fibrosis remained unaltered.

Ulceration and a moderate to severe hyperkeratosis and hyperplasia were observed in the gingival epithelium of treated rats killed at 13 and 14 months but the lesions were much less severe in rats killed after a treatment-free period of 4 months. The epithelium of the tongue and buccal mucosa in treated rats was slight to moderately hyperplastic at all time points of observation.

The artificial lip canal was subsequently employed to explore the possible carcinogenicity of snuff in the oral cavity of rats (Hecht, Rivenson et al, 1986). 50mg of snuff, obtained commercially, was inserted into the lip canal from the oral cavity side using the cap of a JELCO catheter placement unit and a steel plunger. Insertion was made five times weekly for up to 116 weeks in a group of 32 male F344 rats. The solid remaining after extracting snuff with water and filtering (extracted snuff) was air-dried and inserted into the lip canal of 21 rats daily in the same way for the same number of weeks. The filtrate was lyophilised and then brought to the consistency of moist snuff by the addition of water. It was then mixed with commercial snuff and 50mg of this mixture (designated enriched snuff) was inserted once daily in the lip canal for 116 weeks in a group of 32 rats. Ten animals which were subjected to the surgical construction of the lip canal were left untreated and served as controls. No tumours were observed in the oral cavity of the controls. Two tumours, one of which was malignant, developed in the epithelium of the lip canal and there was one papilloma of the hard palate in the group treated with commercial snuff. In the group treated with extracted snuff one rat had a papilloma of the tongue and another a papilloma of the hard palate. One animal in the group treated with enriched snuff developed a papilloma of the floor of the mouth (see Table 1). No sarcomas were reported. The authors state that the incidence of tumours in snuff-treated rats was not statistically significant compared to the controls although the incidence was higher than in some other studies with F344 rats.

Non-specific lesions of the oral cavity including hyperkeratosis, acanthosis, chronic or acute inflammation and granulomas were observed.

Comment

The artificial lip canal model has some merit in the investigation of oral carcinogenesis in that the canal is readily accessible for insertion of test materials and saliva enters the canal from the mouth in copious amounts. Furthermore, absorption of eluates, exemplified by nicotine absorption (Hirsch and Thilander, 1981) may take place readily from the lip canal so that any carcinogenic substances eluted from the test material may be absorbed and may have the opportunity of reaching distal organs.

Unfortunately, however, there is a major drawback in the model which raises substantial doubts about the value of the results obtained. This drawback is the fact that the surgical procedure itself creates a marked inflammatory response. Although the inflammatory response subsides within 2 to 3 weeks of the operation, it leaves a mildly hyperplastic epithelium with minimal amounts of scar tissue formation (Hirsch and Thilander, 1981). In the study by Hecht, Rivenson *et al* (1986) the insertion of snuff was begun three weeks after surgery and this will have reactivated the inflammatory process resulting in

marked epithelial hyperplasia and a progressive increase in scar tissue which would have persisted so long as treatment continues (Hirsch and Johansson, 1983). It is well known that proliferative reactions of this sort in epithelial or connective tissue are prone to lead to cancer induction in laboratory animals even in the absence of any chemical carcinogen (Anderson et al, 1991; Clayson et al, 1991; Poynter and Selway, 1991, Ingram and Grasso, 1991).

The number of epithelial tumours in the lip canal studies is low and a high proportion of them is benign. This picture is well in keeping with the low incidence of tumours in mouse skin or bladder epithelium in mice and rats produced by irritant materials. The fact that no tumours were found in an experiment which lasted 14 months and in which treatment was carried out twice daily for 13 months (Hirsch et al, 1986), suggests that the tumours observed in a subsequent investigation by Hecht et al (1986) may have originated after 13-14 months treatment. A latent period of this sort of duration is in keeping with that observed in the experimental induction of tumours by agents inducing persistent tissue injury (Grasso, Sharratt and Cohen, 1991). Furthermore, the experiments with snuff in the lip canal are lacking in adequate controls. For example, no biologically inert material such as cotton wool was inserted in the controls. Instead, the control group was not subject to any further manipulation while the test animals were subjected to the repeated application of native or modified snuff, a procedure which must involve considerable trauma judging by the severe epithelial and connective tissue reaction that is provoked by it (Hirsch and Johansson, 1983).

Thus, the investigations using the artificial lip canal model have not shown that snuff possesses any carcinogenic activity. In our view, the marked reactive lesion seen histologically in the lip canal following repeated insertion of snuff is sufficient to account for the type and number of tumours observed.

2.5 Promotion studies with smokeless tobacco

The notion of an agent incapable of inducing tumours in its own right but capable of doing so if applied to tissue cells already "primed" or "transformed" by low doses of a strong carcinogen, arose from the early studies on the mechanism of cancer production on mouse skin by PAH carcinogens. The two-stage process if often referred to as the initiation and promotion model of carcinogenesis. The carcinogenic PAHs produced skin tumours within a few months or earlier when applied repeatedly to mouse skin, whereas croton oil and some other substances failed to produce any tumours when tested by the same regimen. If the PAH was applied only a few times at very low doses no tumours resulted but the cells of the skin were "initiated". Subsequent repeated croton oil treatment (promotion) resulted in a high

incidence of tumours. The "initiation/promotion" model has now been shown to occur in liver and urinary bladder in both rats and mice as well as in the skin of mice.

The artificial lip canal model was used to investigate the possible "promoting" effect of snuff following administration of an "initiating" dose of NQO (4-nitroquinoline-N-oxide), a potent topically active synthetic carcinogen on the tongue and hard palate (Johansson, Hirsch et al, 1989). A group of 150 rats had an artificial lip canal created surgically in the lower lip. These were divided into five approximately equal groups.

- Group I were treated with 50 mg snuff inserted twice daily, 5 days a week for 104 weeks;
- Group II were treated by painting propyleneglycol (PG) on the hard palate 3 times weekly for 4 weeks and then left for 104 weeks without further treatment;
- Group III were treated by painting on the hard palate a 0.5% solution of NQO in PG 3 times weekly for 4 weeks but without further treatment;
- Group IV were treated as Group III except that the hard palate painting was followed by the insertion of snuff into the lip canal twice daily for 104 weeks;
- Group V were treated by insertion into the lip canal a cotton dipped in physiological saline 5 days a week for 104 weeks.

There was a low incidence of epithelial tumours in all treated groups but none in the control groups II or V (Table 2). Most of the epithelial tumours were benign and occurred mainly on the hard palate and tongue. The authors concluded that there was no evidence of any promoting effect by snuff because the tumour incidence in the group treated with NQO was the same as that observed in the group treated with NQO followed by snuff. In addition, 5 sarcomas of the lip were observed - 2 in rats treated with snuff alone and 3 in the group treated with NQO followed by snuff.

The possibility that snuff could promote the carcinogenicity of NQO was studied further by Larsson, Johansson et al (1989) in an initiation/promotion experiment combined with an investigation of the interaction of snuff with viruses (see Section 6.3.2 of this monograph and Table 27). In the NQO part of this experiment male inbred Lewis rats were used but in the virus part, including controls and the group treated with snuff only, Sprague-Dawley rats were used. Thus the NQO part of the study lacked controls and a comparable group treated with snuff only. The results showed low incidences of carcinomas in and near the lip canal in the groups treated with NQO only and NQO followed by snuff. There was no significant difference between these incidences and therefore this is further evidence that snuff does not promote the carcinogenicity of NQO.

Johansson, Saidi et al (1991) also used the lip canal model to explore further the possible carcinogenicity of snuff and its ability to promote tumour formation following initiation by either NQO or DMBA. In this study 230 male Sprague Dawley rats had an artificial canal constructed in the lower lip. Forty of these rats (Group I) were treated by insertion into the lip canal of a cotton pellet soaked in a 0.1% solution of DMBA in mineral oil 3 times weekly for 4 weeks and thereafter they received a cotton pellet dipped in physiological saline once a day, 5 days a week for 104 weeks. Another group of 40 rats were treated in the same way with DMBA but then received 50mg snuff twice daily, 5 days a week for 104 weeks (Group II). The next group of 38 rats (Group III) received only snuff twice a day, 5 days a week for 104 weeks. Group IV consisted of 40 rats treated with cotton pellets dipped in a 0.5% solution of NQO in PG 3 times weekly for 4 weeks and then with a cotton pellet dipped in PG once a day for 5 days a week for 100 weeks. Group V (38 rats) were treated with NQO as in Group IV and then with snuff as in Group III. Controls consisted of 30 rats treated with cotton pellets dipped in physiological saline (see Table 3).

Sarcomas occurred in the lip canal in all groups except the one treated with DMBA only (Group I). The highest incidence occurred in the group treated with NQO followed by snuff. In the experiment there was a small number of tumours of the oral epithelium - mostly malignant - in groups II, III, IV and V but the authors concluded that there was no significant difference among these groups in the incidence of these tumours and that no promotional effect was observed. The incidence of tumours outside the head, neck or gastrointestinal tract was higher in the two groups treated with DMBA than in the other groups.

Comment

In the three studies reviewed in this section, epithelial tumours occurred in different treatment groups including those groups treated with snuff only. These tumours were found not only in the lip canal but also on the tongue and hard palate. The incidence is at or below the level of statistical significance if the tumours in each anatomical region are analysed separately against the negative controls. It achieves statistical significance in one experiment by Johansson, Hirsch et al, 1989, if tumours from the hard palate, lip canal, forestomach and nasal cavity in Group 1 are added together and the combined incidence compared with the zero incidence in the controls (Group II). Although such an approach has been favoured by NTP, there are well reasoned arguments against it (Board of Scientific Counselling, 1984) so that evidence of this sort is not regarded as valid proof of carcinogenicity.

The number of epithelial tumours induced by snuff insertion in rats pretreated with DMBA is low and of the same order as in rats treated by snuff alone so that snuff did not act as a promoter in this part of the study. Similarly snuff did not `promote' tumours in animals pretreated with NQO, since there was no difference in the epithelial tumour incidence between groups treated with NQO alone and those treated with NQO + snuff in all three experiments. The high incidence of epithelial tumours in one of the experiments (Johansson, Saidi et al, 1991) is probably due to the relatively high dose of NQO given (five and eight times the amount of NQO in the other two studies). The forestomach lesions seen in the studies reviewed in this section, including a low incidence of carcinomas, probably reflect the irritant properties of the high doses of snuff used. These lesions have no relevance to carcinogenicity at lower exposure levels since it has been shown that forestomach tumours in rodents are caused by a number of substances which are not generally regarded as carcinogens but which produce sustained high levels of cellular proliferation in the rodent forestomach (Clayson et al, 1991).

Connective tissue tumours developed almost entirely around the lip canal. A previous study of the lesions in the connective tissue surrounding the lip canal induced by repeated snuff insertion revealed an active and progressive fibrous tissue reaction which persisted even when treatment was stopped (Hirsch, Larsson et al, 1986). Reactions of this sort are known to be particularly prone to lead to sarcoma production even in the absence of any chemical carcinogens (Grasso, Sharratt and Cohen, 1991). The fact that sarcomas have been reported in the two studies in which a spatula was used to insert the snuff is relevant since its repeated insertion is likely to cause severe local injury. The relatively high incidence of sarcomas in the study by Johansson, Saidi et al (1991) is probably due to a severe connective tissue reaction in response to trauma occurring in tissues pretreated with NQO - a "potent intraoral carcinogen". Fibroblastic proliferation, which is at the heart of this connective tissue reaction, acts as a strong stimulus for tumour production or tumour promotion in the same way as hyperplasia in epithelial tissues (Grasso et al, 1991). The frequent and prolonged use of an instrument such as a spatula would inflict sufficient physical trauma to account for the sarcomas observed.

Thus in these studies the severe mechanical trauma induced by the frequent cleaning and filling of the lip canal can account both for the epithelial and connective tissue tumours induced by snuff insertion. There is no indication that snuff promotes epithelial tumour formation after initiation by DMBA or NQO. There is some evidence that snuff may promote the formation of sarcomas after NQO initiation but it is probable that these tumours were instead "promoted" by the reactive lesion induced by physical trauma.

3 LABORATORY ANIMAL CARCINOGENICITY STUDIES ON TOBACCO-SPECIFIC NITROSAMINES (TSNAs)

3.1 Introduction

Processed tobacco contains a number of nitroso compounds of both the low molecular weight (volatile) and the higher molecular weight (non-volatile) type, derived by the interaction of nitrate/nitrite and amines found in tobacco (Tricker and Preussmann, 1991). The low molecular weight (volatile) nitrosamines are of the type found in several foods such as fish, cured meat and cheese (UK MAFF, 1992) and are present in relatively small amounts in tobacco so that attention has been directed in this monograph almost exclusively to certain non-volatile nitrosamines. These nitrosamines are derived from the secondary amines, nornicotine, anatabine and anabasine and from the tertiary amine, nicotine and are called Tobacco-Specific Nitrosamines (TSNAs). The amounts of TSNAs present varies widely with the nitrite content and type of tobacco (Fischer *et al*, 1989) and are typically present in the order of a few milligrams per kg (Hoffman and Adams, 1981). The following data are from Brunnemann and Hoffmann (1991).

TSNAs in smokeless tobaccos [a]

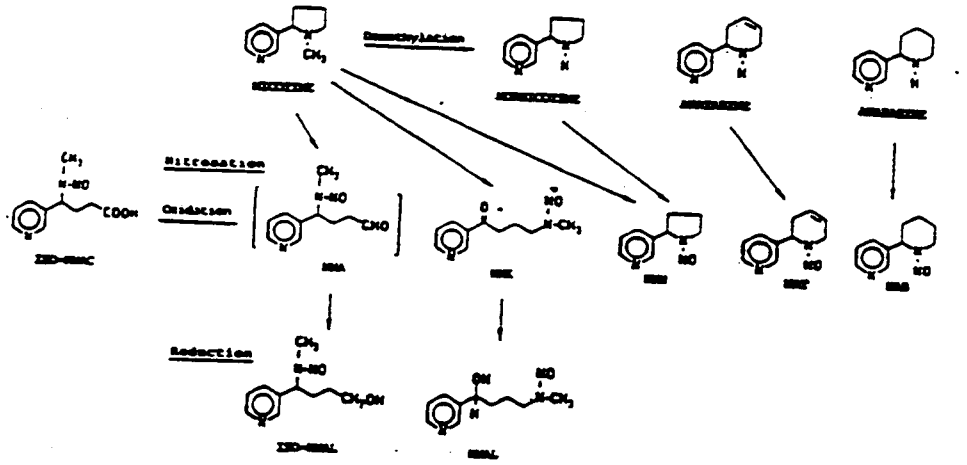
	Product type	NNN	NAT + NAB	NNK	iso-NNAC	Total TSNA
US	moist snuff A	10.4	9.8	2.2	0.1	22.5
US	moist snuff B	9.6	7.9	3.1	0.2	20.8
New US	moist snuff C	4.1	3.0	1.2	0.1	8.4
New US	moist snuff D*	57.1	91.5	7.2	10.5	166.3
Sweden	moist snuff A	5.7	3.5	2.1	0.1	11.4
Sweden	moist snuff B	5.3	2.9	1.4	0.1	9.7
Sweden	moist snuff C	5.2	2.6	1.4	0.1	9.3
US	dry snuff	10.6	13.1	0.9	0.1	24.7
US	chewing tobacco*	1.5	0.7	0.1	0.01	2.3

* same brand name

Key

[a] = Values are based on dry weight.
NNN = N-nitrosonornicotine
NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NAT = N-nitrosoanatabine
NAB = N-nitrosoanabasine
iso-NNAC = 4-(methylnitrosamino)-4-(3-pyridyl)-4 butyric acid

The following is a diagrammatic representation from Brunnemann and Hoffmann (1991) of the formation of the main TSNAs from their parent alkaloids.



Four TSNAs (NNK, NNN, NAB and NAT) have been tested for carcinogenicity.

3.2 Studies with NNK

NNK has been investigated for its carcinogenic potential in laboratory animals by various routes of administration.

3.2.1 Topical Application

In mice, NNK was applied topically to the skin of the Sencar strain at 0.03, 0.1 or 0.6 mg/mouse/day for 10 days and was then followed by a weekly application of tetradecanoylphorbol acetate (TPA) to the same site for a further 20 weeks. Local proliferative skin lesions developed but their identity is uncertain since no histological examination was carried out (La Voie *et al*, 1987; Table 7). Topical application of NNK to the ventral side of the tongue in two strains of male mice (Balb/c and Sencar) for 7 weeks did not produce any local tumours but tumours of liver and lung were observed in treated groups only (Padma, Lalitha *et al*, 1989; Table 7).

In a group of 30 rats, oral papillomas (6 of the cheek epithelium, 1 of the hard palate and 2 of the tongue) developed after twice daily oral application of an aqueous mixture containing 14µg NNK and 68µg NNN. The dosing was continued for up to 2½ years when the experiment terminated (Hecht, Rivenson *et al*, 1986; Table 11). Only one oral papilloma was observed in a 61-week study on 29 rats following

topical application of an aqueous solution of NNK twice daily at approximately 1mg per application (Prokopczyk, Rivenson and Hoffmann, 1991; Table 11).

In hamsters, cheek pouch papillomas developed in 4% of survivors following thrice weekly local application of 1mg NNK to groups of 30-40 animals for 40 weeks. The experiment was terminated at 90 weeks. Local application of 1mg NNK twice daily for 5 days did not produce tumours at 66 weeks when the experiment was terminated (Padma and Lalitha *et al*, 1989; Table 14).

3.2.2 Parenteral Dosing

In studies in female A/J mice intraperitoneal administration of 0.2-1 mg/mouse of NNK in tricaprylin or in saline three times weekly for 33-37 weeks resulted in the production of a statistically significant increase in pulmonary tumours the majority of which were benign (Table 6). A single injection of 0.2-2 mg/mouse NNK produced a dose-related increase in lung adenomas 16 weeks later when the experiment was terminated (Peterson and Hecht, 1991). A statistically significant increase in lung adenomas was found in 9 experiments when female mice were treated once only with 2mg/mouse NNK. The control mice were treated with the dosing vehicles. These studies were designed to explore the inhibitory effect of a variety of chemicals or type of diet to the carcinogenicity of NNK- It was found that more tumours developed in mice maintained on semi-synthetic diet from weaning than in those on a normal diet (Hecht, Morse, Amin *et al*, 1989). Alkylarylisothiocyanates reduced the tumorigenicity of NNK (studies by Morse and co-workers published betwen 1989 and 1992). Indole-3-carbinol and deuteration of the methylene carbon to the nitroso group had a similar effect (Morse, La Greca *et al*, 1990; Hecht, Morse, Eklind, Chung, 1991).

In rats, subcutaneous administration of NNK in trioctanoin consistently induced tumours of the lung, liver and nasal cavity in five experiments (Table 10). Thrice weekly injection of 12mg/rat NNK (approximately 36mg/kg body weight) for 20 weeks produced over 85% incidence of these tumours in male rats by the termination of the study at 52 weeks. No tumours were observed in controls (Hecht, Chen and Ohmori, 1980). A dose response relationship was observed in the development of these tumours when male rats were injected 3 times weekly with 3.3, 10 or 30 mg/kg body weight of NNK for 20 weeks and the experiment terminated at 120 weeks (Hoffmann, Rivenson *et al*, 1984). A similar dose-response was observed in the induction of these tumours when rats were given 0.03, 0.1, 0.3, 1, 10 or 50 mg/kg body weight three times weekly for 20 weeks and the experiment terminated at 120 weeks (Belinsky, Foley *et al*, 1990; Belinsky, Devereaux *et al*, 1991).

Deuteration of the methyl carbon α to the nitroso group did not reduce the tumour incidence produced by the subcutaneous injection of NNK at dose-levels and duration similar to those described in the previous subcutaneous studies while dietary phenylethylisothiocyanate (PEITC) reduced the incidence of lung tumours by approximately 30% (Table 10). PEITC was chosen because previous studies had shown that it affected the metabolism of NNK in a manner which suggested that it was a potential inhibitor of NNK carcinogenesis.

Hamsters dosed subcutaneously with 10mg/hamster NNK thrice weekly for 6 weeks developed incidences of 50% lung adenomas and 10% lung adenocarcinomas as well as 50% nasal carcinomas by the termination of the experiment at 65-70 weeks. In a second group in which hamsters were given 2.5mg NNK/hamster three times weekly for 25 weeks the incidence of these tumours was 60%, 20% and 50% respectively. Thirty-five percent (35%) surviving hamsters also had tracheal papillomas by 65-70 weeks (Table 13). The same histological types of tumours were observed when hamsters were given NNK subcutaneously at 1, 3.3 or 10 mg/hamster and killed 1½ years later. There was no quantitative dose response relationship but none of these tumour types were seen in controls.

3.2.3 Gavage and drinking water studies

In mice (Swiss strain), administration of an aqueous solution of NNK by gavage at a dose of 1mg/mouse/day (approximately 25mg/kg body weight) for 22 days produced a high incidence of lung adenomas (approximately 80%) in animals that were kept until their deaths when they were 17-25 months old or at the termination of the study at 68-108 weeks. No tumours of the oral cavity or upper alimentary tract were observed (Bhide, Kulkarni *et al*, 1989; Table 8). Likewise, a high incidence of lung adenomas was produced in mice of the A/J strain when NNK was given in drinking water at a dose of 3 or 9 mg/mouse (approximately 75-225 mg/kg body weight) continuously for the first 7 weeks of an experiment of 23 weeks duration (Castongay, Pepin, Stoner, 1991).

In rats, NNK produced a dose-related incidence of tumours of the lung, nasal cavity and liver when given in the drinking water at 0.5, 1 or 5 ppm for 2 years but there were no oral or upper alimentary tract tumours (Rivenson, Hoffmann *et al*, 1988; Pour and Rivenson, 1989; Table 12). A similar result was obtained in a later study by Lijinsky *et al* (1991) in which NNK was administered by gavage.

3.3 Studies with NNN

3.3.1 Topical application

In mice, 10 topical applications of 0.5mg NNN dissolved in acetone on alternate days was followed by thrice weekly applications of tetradecanoylphorbol acetate for 20 weeks. The experiment was terminated at 24 weeks. Two local tumours (type not identified) were observed in the treated group and one tumour in the control group (La Voie *et al*, 1987; Table 18). No tumours developed in the oral cavity of Swiss or Balb/c mice when 1 mg of NNN in aqueous solution was applied thrice weekly to the ventral side of the tongue for 8 weeks (Swiss strain only) or 24 weeks (both strains). The experiment was terminated at 90 weeks. An increased incidence of pulmonary and hepatic tumours was observed in Swiss and Balb/c mice treated for 24 weeks (Padma, Lalitha *et al*, 1989; Table 18).

In one experiment in rats a few local benign oral tumours were observed when an aqueous mixture of NNN and NNK was applied to the lips and oral cavity (see Section 3.2. 1).

3.3.2 Parenteral dosing

In studies on female A/J mice dosed intraperitoneally 3 times weekly for 7 weeks with approximately 1mg of NNN per dose the percentage of mice with lung tumours ranged from 48-83% (Table 16). The duration of the experiments was 33-37 weeks. The range of tumour incidence in controls varied from 12-40%. Most lung tumours were classified as adenomas. In three studies, NNN was administered in saline (Hecht, Chen, Hirota *et al*, 1978; Castonguay, Lin *et al*, 1983; Hecht, Abbaspour and Hoffmann, 1988) and in one study in trioctanoin (Hecht, Chen, Hirota *et al*, 1978). A high incidence of lung adenomas was also observed in A/He mice given approximately 1mg/mouse NNN 3 times weekly for 8 weeks and terminating the study at 32 weeks (Hoffmann, Hecht *et al*, 1976; Table 17).

In three studies in rats repeated subcutaneous injection of NNN induced predominantly tumours of the nasal cavity (Table 19). A low incidence of oesophageal and lung tumours was also produced. In the first study, a dose of 10mg/rat (approximately 30mg/kg body weight) of NNN in trioctanoin was given 3 times weekly for 20 weeks and the experiment terminated at 52 weeks. The only compound-related tumours were in the nasal cavity (Hecht, Chen, Ohmori and Hoffmann, 1980). In a second study three doses of 27, 9 and 3 mg/kg body weight were employed. A high and dose-related incidence of malignant nasal cavity tumours and of benign oesophageal tumours was observed (Hoffmann, Rivenson *et al*, 1984). In hamsters, intraperitoneal administration of NNN dissolved in saline 3 times weekly for 25

weeks at 2.4 or 4.7 mg/hamster produced a dose-related incidence of tumours of the nasal cavity and trachea by the time the experiment was terminated at 78 weeks. Liver tumours were also observed and were treatment-related (McCoy, Hecht *et al*, 1981; Table 21). Subcutaneous administration of NNN in saline at a dose of 5mg/hamster 3 times weekly for 25 weeks produced mainly tracheal tumours and one nasal carcinoma (Hilfrich *et al*, 1977; Table 22). Administration of NNN in trioctanoin at 8.6mg/hamster for 6 weeks or 2.2mg/hamster for 25 weeks produced a compound-related incidence of tracheal papillomas and lung adenomas (Hoffmann, Castonguay *et al* 1981; Table 22).

3.3.3 Oral administration

In mice, when an aqueous solution of NNN was given by gavage (1mg/mouse, 5 days/week) for approximately 5 weeks a higher incidence of forestomach, lung and liver tumours than controls were found at the termination of the experiment 1.5-2 years later (Bhide, Kulkarni *et al*, 1989).

In rats NNN added to the drinking water produced a relatively high incidence of oesophageal tumours as well as tumours of the nasal cavity and a low incidence of tracheal papillomas (Table 20). In the first of these studies (Hoffmann, Raineri *et al*, 1975; Hoffmann, Hecht and Onaff *et al*, 1976) male Fischer 344 rats were given a total of approximately 630mg NNN/rat over 30 weeks. The experiment was terminated at 47 weeks. Out of the 20 exposed rats, 14 had oesophageal tumours of which 3 were malignant, 3 had nasal carcinomas and one had a pharyngeal papilloma. In a second study (Hecht, Young and Maeura, 1983) 24 Fischer 344 rats were given total doses of approximately 637mg/rat (males) and 584mg/rat (females) over 36 weeks. At the end of the study (43 weeks) 23 rats had oesophageal papillomas and 6 of these rats had malignant oesophageal tumours as well. Fifteen rats had malignant nasal tumours and one had a tracheal papilloma. No meaningful sex difference was found. A similar incidence of benign and malignant oesophageal and nasal tumours was observed by Castonguay, Rivenson *et al* (1984) at the termination of a study (98 weeks) in which F344 male rats were fed a liquid diet containing NNN continuously for 27 weeks with an estimated total dose of 177mg/rat. No tumours of these types occurred in controls of all three experiments. There was no indication that NNN increased the incidence of commonly occurring tumours.

In hamsters (Table 21) given NNN in drinking water continuously at an approximate total dose of 336mg (males) or 496mg (females) per hamster for 31 weeks there was a combined incidence of 20% nasal and 10% tracheal papillomas at 96 weeks when the study was terminated. An animal developed a hepatic angiosarcoma (Hecht, Young and Maeura, 1983; Table 21).

3.4 Studies with NAT

NAT was tested by twice weekly subcutaneous injections for 20 weeks in Fischer 344 rats with total doses up to 1700mg/kg body weight. The rats were then left untreated until the experiment was terminated at 2 years. No tumours were induced (Hoffmann, Rivenson *et al*, 1984).

3.5 Studies with NAB

NAB was administered in drinking water in two experiments in rats (Table 24). Oesophageal tumours were produced in the Chester Beatty rat after continuous treatment for 74 weeks (Boyland *et al*, 1964). In the other experiment, treatment was terminated at 48 weeks and only one oesophageal and one pharyngeal papilloma were observed (Hoffmann, Raineri *et al*, 1975). No oesophageal tumours were observed in controls of either experiment.

No tumours were produced when NAB was injected subcutaneously three times weekly for 25 weeks with a total dose of approximately 375mg/hamster. The duration of the study was 83 weeks (Hilfrich *et al*, 1973).

3.6 Comment

Most nitroso compounds are carcinogenic in one or more species of laboratory rodent and they tend to produce tumours in more than one organ. NNK and NNN behave like most other nitroso compounds (including those found in food) in this respect. It is important to point out that the oral cavity is not one of the target organs for NNK and NNN.

In mice, NNK and NNN produce mainly lung tumours irrespective of the method of administration although in one experiment, topical application of NNK produced tumours in liver in addition to pulmonary tumours. Presumably because of the high dose of NNN or NNK employed in these experiments virtually all treated mice developed tumours even though the experiments lasted a few months. Swiss mice of the A/J strain are known to be genetically susceptible to the development of pulmonary adenomas under natural conditions. Induction of these tumours readily follows administration of carcinogenic agents so that the mouse experiments are of little value in assessing the carcinogenic potency of NNN or NNK.

In rats, the principal target organ for both NNK and NNN is the nasal cavity. Both compounds produce a high incidence of tumours in this organ when administered by the oral or parenteral route. In addition NNK produces tumours of the liver and lung while NNN produces tumours of the oesophagus. This pattern of tumour production is the same whether the TSNAs are administered parenterally or orally but oral administration of NNN produces a higher incidence of oesophageal tumours than the parenteral route.

The anatomical sites at which tumours appear possess enzymes of the cytochrome P450 type which are capable of metabolising these two TSNAs to short-lived proximate carcinogens. Other tissues may deactivate TSNAS. These effects vary depending on the route of administration. By some routes the TSNAs quickly reach tissues which activate them. By other routes they are exposed to tissues containing deactivating enzymes (e.g. pyridyl N-oxidising enzymes) before they reach the tumour-susceptible tissues. This would account for the fact that NNN produces more oesophageal tumours when given orally than when given parenterally. This activity is not typical of classical contact carcinogens which affect the primarily-contacted tissues irrespective of enzyme activity.

It is important to note that no oral tumours were produced in rats or mice by either parenteral or oral administration of NNK or NNN dosed separately. This suggests that the oral epithelium does not possess the ability to produce the proximate carcinogenic metabolites even at the high doses administered.

A few oral papillomas were observed in a study in rats with a topically applied mixture of NNK and NNN and in studies in rats and hamsters with NNK alone. In these studies the oral mucosa was repeatedly swabbed with the TSNAs for long periods and it is likely that the oral papillomas were the result of repeated mechanical trauma combined with repeated exposure to lipophilic chemicals. There is strong evidence that such treatment produces massive hyperplasia at the site of application and that hyperplasia in rodents may lead to the formation of papillomas. The papillomas did not progress to malignant tumours in spite of the long duration of the studies and the high frequency of exposure. This lack of progression suggests that the "papillomas" could be focal hyperplastic rather than neoplastic lesions.

4 METABOLISM AND TOXICOKINETICS OF NNN AND NNK

By the oral route, NNN and NNK are almost completely absorbed from the intestine since only minor proportions of the administered dose are found in the faeces (Castonguay, Tjalve and Hecht, 1983). The

major route of excretion of metabolites of NNK is via the urine although when the ^{14}C label is on the CH_3 group substantial amounts of $^{14}CO_2$ is exhaled via the lung due to cleavage of this part of the molecule.

^{14}C labelled material was widely distributed throughout the body with high levels in liver, nasal mucosa and kidneys, following parenteral administration of 5mg/kg body weight ^{14}C-labelled NNN and NNK to rats. Similar results were obtained with hamsters and marmosets (dose not stated). Accumulation was also observed in pigmented tissues such as eye and skin and in some exocrine glands (Tjalve, 1991).

NNN and NNK are asymmetric N-nitrosamines so that they can each form more than one alkylating agent. Their major route of metabolic activation involves an initial cytochrome P450 dependent α-hydroxylation with ultimate formation of an alkylating species. Hydroxylation of the methyl carbon of NNK and at the 2' position in the pyrrolidine ring of NNN results in a common metabolic pathway. The alkylating agent formed by this pathway is thought to be the 4-(3-pyridyl)-4-oxo-1-butylcarbonium ion (Structure [1] in figures 1 and 2). NNK is also metabolised to form a methylcarbonium ion, another alkylating species. An alternative route of NNN activation results in formation of 4-formyl-1-(3-pyridyl)-1- butylcarbonium ion.

Other routes of metabolism exist for NNN and NNK which appear to represent, predominantly, detoxification pathways.

4.1 Metabolism by different tissues

4.1.1 *In vivo*

Metabolism of NNN and NNK in specific tissues has been investigated *in vivo* by autoradiographic studies of sections extracted to remove unbound radiolabelled material. It was assumed that, since the reactive intermediates produced from these nitrosamines have very short half-lives, they bind to tissue macromolecules which are in close proximity to the site of initial α-hydroxylation (Tjalve, 1991).

Parenteral injection of carbonyl-^{14}C-NNK in rats resulted in the accumulation of bound metabolites in the upper respiratory tract and the liver. Oral administration resulted in the labelling of the oral and oesophageal mucosa as well (Tjalve, 1991).

Injection of ^{14}C-NNN in rats showed accumulation of bound metabolites in the upper and lower respiratory tract, liver, Bowmans glands of the olfactory mucosa and oral/oesophageal epithelium

(Tjalve, 1991; Tjalve and Castonguay, 1987). Labelling patterns in mice and hamsters injected with NNN or NNK were similar to those seen in rats but marmoset monkeys accumulated bound metabolites only in liver and nasal mucosa.

4.1.2 *In vitro*

NNK is efficiently metabolised by microsomal preparations from nasal and liver tissues of rats, mice and hamsters. The relative proportions of α-hydroxylation (activation routes) to N- oxidation (detoxication) have been reported as 386x in liver and 6x in lung microsomes (Castonguay, Pepin and Stoner, 1991).

In freshly isolated F344 rat hepatocytes the major metabolite of NNK is NNAL (Liu, Moulay *et al*, 1990). In freshly isolated lung cells from F344 rats metabolite production was highest in Clara cells, which contain the greatest content of cytochromes P450. N-oxidation occurred at about half the rate of α-hydroxylation in Clara cells and alveolar type II cells but was not detectable in alveolar macrophages. Carbonyl reduction to NNAL predominated over the other metabolic pathways in all three cell types (Belinsky, White *et al*, 1989). α-hydroxylation was the major route of NNK metabolism of Clara cells from Syrian hamsters: carbonyl reduction to NNAL was predominant in alveolar macrophages (Alaoui-Jamali *et al*, 1990).

Organ culture of lung trachea from hamsters revealed that the major metabolism pathway of NNK was via α-hydroxylation rather than N-oxidation whereas in similar preparation of rat lung (F344) the major pathway was via N-oxidation (Castonguay, Allaire *et al*, 1989; Doerr-O'Rourke *et al*, 1991).

No N-oxidation was detected when NNN was incubated with Clara cells, type II macrophages and alveolar macrophages, otherwise the pattern and rate of metabolism by these cells was the same as those of NNK (Belinsky and White *et al*, 1989). Results of these studies revealed that the labels of the alkylating species common to both NNN and NNK (butyl carbonium ion) formed in lung cells from NNN are not substantially less than those formed from NNK suggesting that the lung tumours produced by NNK in rats are formed from the reactive moiety derived from the other pathway of metabolism (methyl carbonium ion).

α-hydroxylation of NNN by explants of rat oesophagus was greater than that from NNK in studies conducted under comparable conditions. Carbonyl reduction and N-oxidation (detoxification) were the major routes of NNK metabolism in oesophageal tissue. This would suggest that α-methylene

hydroxylation of NNN is mainly responsible for the production of oesophageal tumours by this agent (Murphy *et al*, 1990).

A study conducted on human tissues indicated that α-hydroxylation of NNN and NNK and carbonyl reduction of NNK occurred in a variety of tissues at 1-10% of the level seen in animal tissues (Castonguay, Stoner *et al*, 1983).

4.2 Modulation of TSNA metabolism

Inhibition of metabolism of TSNAs by various components of certain fruits and vegetables is summarised in combination with consideration of the effects on genotoxicity (section 5.5). The effect of tobacco constituents on the metabolism of TSNAs has also been investigated. Metabolism of NNK was inhibited only slightly by NNN in cultured rat oral tissue, whereas NNK was much more effective in inhibiting the metabolism of NNN (Murphy and Heiblum, 1990), suggesting that different enzymes are responsible for the metabolism of NNN and NNK. N'-Nitrosoanatabine (NAT) inhibited the metabolism of NNK, but not of NNN. Nicotine caused an appreciable inhibition of all routes of NNN metabolism, even at a 1:1 ratio, when the level of most metabolites decreased by 90% (Murphy and Heiblum, 1990). Nicotine inhibited α-hydroxylation and N-oxidation, but not carbonyl reduction of NNK, even when present at 100 times the concentration of NNK (Murphy and Heiblum, 1990). Similar effects were reported to occur in hamster lung cultures (Schuller, Castonguay *et al*, 1991). Effects on the conversion of NNAL to alkylating species were not investigated in these studies. Prolonged treatment of hamsters with 0.002% nicotine in the drinking water induced both α-hydroxylation and N-oxidation of NNK in hamster lung cultures (Charest *et al*, 1989). Model inducers of cytochrome P450 (phenobarbitone, 3-methylcholanthrene and butylated hydroxyanisole) induced only the α-hydroxylation route (Charest *et al*, 1989).

4.3 Comment

Toxicokinetics and metabolism data are thought to offer a means for obtaining an understanding of the mechanism by which chemicals produce their biological effects and hence the practical significance of animal carcinogenicity studies.

The toxicokinetics studies outlined in this section reveal that on parenteral or oral administration NNN and NNK are not only taken up by the organs which develop tumours on prolonged administration but

they are also taken up by other tissues so that the kinetic data offer no assistance in explaining the reason for the development of tumours in certain specific sites.

Metabolism studies appear to be somewhat more useful in this respect. First of all detailed studies of metabolism pathways indicate that two types of alkylating species (methyl carbonium ion and butyl carbonium ions) are produced which bind with protein and have the potential for damaging the DNA leading to the conclusion that the tumours produced are likely to be the results of a genotoxic mechanism.

Attempts to identify the sites at which these reactive intermediates occur revealed that they occur at the 'target' organs and at others which were not affected by tumour production. For example carbonyl ^{14}C-NNK accumulated in organs which produced tumours (respiratory tract and liver) as well as in organs which were spared (oral and oesophageal). Similarly NNN metabolites not only bound to nasal and oesophageal mucosa but also to lung and tracheal tissues which were not the targets in tumour formation. Thus in vivo metabolism studies have also failed to establish a clear correlation between local binding of radiolabelled metabolites and tumour formation.

In vitro studies provide further examples of such apparent anomalies. Thus the proportion of the activation route compared to the deactivation route of NNK in mouse liver was much higher than that in the mouse lung. Yet tumours are produced in lung but not in liver of mice given NNK. Although these sorts of anomalies preclude the use of results from metabolic studies to make extrapolation to another species they have been useful in clarifying some minor points. Thus it would appear that lung tumours are caused by the methylcarbonium ion which is a major alkylating product of NNK. Likewise, a greater degree of local binding was observed in oesophageal tissue from rats treated with labelled NNN than in the oesophagus from rats treated with NNK correlative with incidence of tumours.

The efforts so far employed in studying the metabolism of NNN and NNK have provided data of limited usefullness for an understanding of metabolism in specific tissues and the relationship of this to cancer development.

5 GENOTOXICITY OF SMOKELESS TOBACCO AND TSNAs

5.1 Mutagenicity *in vitro*

Mutagenicity studies were devised to detect whether a substance could interact with DNA and therefore have the potential to cause cancer. They are generally conducted on substances which have not been

screened for carcinogenicity, as part of the risk assessment process and to determine whether a carcinogenicity study is required. Alternatively, they may be conducted to investigate the mechanism of carcinogenicity of a substance that has already been demonstrated to cause tumours. However, in the case of smokeless tobacco, the mutagenicity studies were conducted after the negative carcinogenicity studies. In such circumstances, the results of the carcinogenicity studies should be afforded more importance in assessing hazard for man.

A thorough investigation of mutagenicity includes assessment of gene mutations in bacteria and mammalian cells, clastogenicity (chromosomal effects) in mammalian cells both in vitro and in vivo, and DNA damage (directly by single strand breaks, or indirectly by DNA repair detected as unscheduled DNA synthesis). Some examples of investigations of each of these types have been reported for either tobacco extracts or certain TSNAs, but the data are not comprehensive. Smokeless tobacco cannot be applied directly to such assay systems: the constituents are extracted into organic solvent or water prior to testing.

5.1.1 Smokeless tobacco

5.1.1.1 *Bacterial systems*

The mutagenicity of smokeless tobacco extracts, has been investigated in bacterial and mammalian cell systems. Aqueous extracts of smokeless tobacco products sold in the USA were found to be mutagenic to Salmonella typhimurium strain TA100 in the presence of hamster liver S9 fraction (Guttenplan, 1987), indicating that constituents of the extract could be metabolised to mutagens which cause point mutations (base-pair mutations). Only weak mutagenic activity was observed in strain TA98, which detects frameshift mutations. The activity was observed in aqueous extracts, but only minimally in ether extracts, demonstrating that the mutagenic components were polar in nature, possibly organic acids and not NNN or NNK which are extractable by organic solvents from aqueous solution (Guttenplan, 1987). The mutagenicity dose response showed an increase in revertants with the extracts from a minimum extract concentration equivalent to 25 mg of tobacco. The potencies of the extracts were in the range of 8-16 x 10^3 revertants of TA100 per g tobacco. This is similar to the reported range of 5-10 x 10^3 revertants of TA100 per g tobacco for cigarette smoke condensates (Guttenplan, 1987).

5.1.1.2 *Mammalian systems*

The mutagenicity of aqueous extracts of American chewing tobacco was investigated in two human cell lines: AHH-1, which expresses cytochrome P450 1A1, and TK-6, which has been shown not to express cytochrome P450 species (Shirnamé-Moré, 1991). The extracts were equally mutagenic to both cell lines at concentrations of 1-3 mg/ml extractable solids. Treatment of the extracts with nitrite at either neutral or acidic pH gave no evidence for the presence of constituents which could be nitrosated to mutagenic substances. These results could indicate that a) the mutagenicity is not cytochrome P450 mediated (as the author concluded), b) P450 1A1 is not the isozyme responsible for metabolising the constituents of these extracts, or c) additional isozymes of P450 are present in both cell lines, that have not yet been detected.

Induction of mammalian cell mutation of TSNAs has been investigated in a human cell line, designated 2D6/Hol, which expresses human cytochrome P450 2D6 (Crespi, Penman *et al*, 1991). Exposure of 2D6/Hol cells to NNK concentrations of 30 - 90 µg/ml induced an increase in mutations at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus. NNK did not induce mutations in control cells (the parent AHH-1 TK +/- cell strain without the transfected P450 2D6 cDNA) at concentrations up to 150 µg/ml. NNN was not mutagenic to 2D6/Hol cells at concentrations of 150 and 300 µg/ml, indicating that P450 2D6 does not activate NNN. Interestingly, the non-carcinogenic NNA was equally mutagenic to 2D6/Hol cells and the AHH-1 TK +/- cells, possibly due to metabolic activation by another form of P450 which was native to the non-transfected cells. NNK also induced mutations in cell lines expressing P450s 1A1, 2A3 and 2E1, with relative sensitivities of 1A2/Hol = 2A3/Hol > 2D6/Hol > 2E1/Hol, although NNN and NNA were not investigated in these lines. Compared to DMN in cells expressing 2E1, NNK was approximately 1000-fold less active as a mutagen on a molar basis in cells expressing each of the 4 isozymes (Crespi, Penman *et al*, 1991).

5.1.2 TSNAs

5.1.2.1 *Bacterial systems*

NNN and NNK, at concentrations in excess of 100µg/plate, were shown to be mutagenic to TA100 in the presence of a metabolic activation system consisting of S9 fraction from Aroclor 1254-pretreated rats. Weaker activity in both NNK and NNN was seen in strain TA1535 (the DNA repair proficient, parent strain of TA100), but not in TA98 or TA1538 at concentrations of 1000µg/plate (Padma, Amonkar and Bhide, 1989).

Primary rat hepatocytes from either control or Aroclor treated rats induced mutagenicity in TA1535, although 10 times more control rat hepatocytes were required to produce a similar response to that seen with Aroclor treated rat hepatocytes (Zhu, Cunningham *et al* 1991).

The relative mutagenic potencies of the methylating and pyridyloxobutylating species have been investigated using acetoxymethylmethylnitrosamine (AMMN), which generates the same methylating species as that formed following methylene hydroxylation of NNK, and 4-(acetoxy-methylmethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc), the pyridyloxobutylating model (Foiles, Peterson *et al*, 1992). NNKOAc was 10 times more potent than AMMN in inducing reverse mutations in Salmonella typhimurium strains TA98, TA100 and TA1535 in studies without activation by the S9 liver preparation.

Characterisation of the mutational specificity of NNK in the *lacI* gene of *Escherichia coli*, demonstrated that 55% of mutations recovered were of the GC to AT transition (Jiao, Zielenska *et al*, 1991). This is consistent with the principal product of DNA alkylation by NNK being O^6- methylguanine (O^6MG). In addition, other base-pair substitutions, frameshift mutations, duplications and deletions were detected. This complexity of mutations suggests that NNK acts through more than one pathway, consistent with a major role for DNA methylation, with a lesser contribution from pyridyloxobutylation (Jiao, Zielenska *et al*, 1991).

5.1.2.2 *Mammalian systems*

The methylating model compound, AMMN (acetoxy methyl methyl nitrosamine), was 5 times more potent than the pyridyloxobutylating agent, (NNKOAc) derived from NNK metabolism, in inducing mutations at the thymidine kinase locus in G12 cells (Foiles, Peterson *et al*, 1992), suggesting that methylation is the more important route in mammalian cell mutagenicity in contrast to the effects in bacteria (section 5.1.1.2), but in agreement with the carcinogenicity data (section 3.2.2).

NNK, at concentrations of 20-200 µg/ml [c. 0.1-1.0 mM], was shown to induce a dose dependent increase in sister chromatid exchange (SCE) in V79 cells in the presence of liver S9 fraction from Aroclor 1254 pretreated rats (Ziminjic, Popescu and DiPaolo, 1989). NNK (up to 80mM) and 4-hydroxy-1-(3-pyridyl)-1-butanone (1mM) a metabolite of NNK (see Figure 1) did not induce SCEs in the absence of metabolic activation (Alaoui-Jamali, Gagnon *et al*, 1990). In contrast, 4-oxo-4-(3-pyridyl)-butyraldehyde, another metabolite of NNK, induced a dose- related increase in SCEs without S9

activation at concentrations of 0.05 to 0.1 mM, indicating that this could be one of the NNK metabolites responsible for the clastogenicity (Alaoui-Jamali, Castonguay and Schuller, 1988).

Induction of DNA single strand breaks (SSBs) by NNK and some of its metabolites has been investigated in V79 cells and in isolated rat hepatocytes. 4-Oxo-4-(3-pyridyl)-butyraldehyde induced SSBs at concentrations of 0.1-1.0 mM in V79 cells (Alaoui-Jamali, Gagnon *et al*, 1990) and at 1-2 mM in hepatocytes, at which concentrations it was also cytotoxic (Demkowicz- Dobrzanski and Castonguay, 1991). This difference in response at comparable dose levels may be due to the superior capacity of hepatocytes for detoxication of this metabolite. The dose- response relationship for SSB and cytotoxicity for the aldehyde in hepatocytes was very steep compared to that of NNK, which induced SSBs at 0.5mM, but was less genotoxic and cytotoxic than the aldehyde at higher concentrations. This observation suggests that the aldehyde may not be the major metabolite responsible for the genotoxicity of NNK (Demkowicz-Dobrzanski and Castonguay, 1991). Two other NNK metabolites, carbethoxynitrosoaminomethane and 4-(N- carbethoxy-N-nitrosamino)-1-(3-pyridyl)-1-butanone (NNC) induced similar levels of SSBs to 5mM NNK at much lower concentrations (5 and 100 µM, respectively) (Liu, Moulay *et al*, 1990).

5.2 Genotoxicity *in vivo*

In vitro mutagenicity assays give an indication of the potential of a substance to be a genotoxic carcinogen. Whether that potential will be expressed in vivo is determined by a complex interaction of factors, such as site and rate of absorption, distribution and accumulation in responsive tissues, relative contribution of activation and detoxication pathways, routes and rates of excretion, etc. These cannot be predicted, and it is therefore necessary to conduct *in vivo* experiments to determine whether a substance will express its genotoxicity. However, it is not feasible to reproduce in vivo the full range of *in vitro* genotoxicity endpoints. Studies of clastogenicity (micronuclei or aberations in metaphase chromosomes) in bone marrow cells of treated rats or mice are most commonly used.

Both NNN and NNK were shown to induce a dose-related increase in bone marrow cell micronuclei of mice treated with 2 daily i.p. doses of 250 and 500 mg/kg body weight of TSNA by a standard protocol (Padma, Amonkar and Bhide, 1989). These doses represented the LD50 and 1/2 LD50, which is in the normal range for this assay. The authors stated that NNK induced a significantly higher number of micro-nuclei compared to NNN, although the data appeared very similar. Percentages of micronuclei, similar to those induced by 250 mg/kg NNN were reported to occur following treatment of mice [route not stated] with 0.25 mg ethanolic tobacco extract (Bhide, Kulkarni *et al*, 1989). Injection of 3 daily i.p.

doses of 50 - 150 mg/kg NNK to F344 rats resulted in induction of micronuclei in tracheal epithelial cells (Zhu, Cunningham *et al*, 1991). Single strand DNA breaks were found in hepatocytes isolated from rats injected s.c. with a single dose of 0.39 mmol/kg NNK (Demkowicz-Dobrzanski and Castonguay, 1991). DNA fragmentation increased to a peak 12 hours after injection, and was repaired in an initial fast phase, then a slower phase, such that no breaks were apparent 2 weeks after injection.

5.3 DNA adduct formation

As noted in section 4, metabolic activation of NNK occurs via hydroxylation of the methylene or methyl carbon, resulting in methylation or pyridyloxobutylation of DNA, respectively. NNN does not produce methyl adducts. The majority of studies of TSNA-induced DNA adducts have concentrated on DNA methylation by NNK. Formation of DNA adducts is generally taken as the most conclusive evidence that a substance can interact with DNA within a target tissue. Detection of methyl adducts requires a radiolabel in the relevant methyl moiety of the chemical under investigation, as many different chemicals can cause methylation of DNA. The pyridyloxobutyl DNA adduct formed by NNK and NNN is detected by hydrolysing the DNA in order to release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, structure [2] in Figures 1 and 2). Detection of DNA adducts is not, however, conclusive evidence that a substance will be mutagenic. Particularly at low levels of adducts and in cell types with low turnover rates, the DNA may be repaired before being replicated and therefore the lesion does not become a mutation. Capacity for DNA repair, and the fidelity of repair varies with level of adducts, type of cell, species and between individuals.

Administration of NNK to rats by various routes resulted in formation of 7-methylguanine (7MG), O^6-methylguanine (O^6MG) and O^4-methyldeoxythymidine adducts of DNA in the liver, lung and nasal mucosa (Hecht, Trushin *et al*, 1986; Belinsky, White *et al*., 1986). Methylation was most extensive in the nasal mucosa, followed by liver, and least in lung. 7MG adducts were approximately 10 times more prevalent than O^6MG adducts, but O^6MG is considered to be more important because it causes mis-pairing during DNA replication, resulting in a G-C to A-T transition. The levels of all 3 adducts increased throughout a 12 day treatment period in the lung, but decreased in the nasal mucosa. The activity of O^6-methylguanine-DNA methyltransferase (O^6MGMT), which reflects the ability to repair the O^6MG adduct, decreased throughout the treatment period in lung and nasal mucosa (Belinsky, White *et al*, 1986).

The concentration of O^6MG in the Clara cells of the lungs of rats treated with NNK correlated directly with lung tumour incidence (Belinsky, Foley *et al*, 1990). This correlation was not observed for whole lung or type II cells, although morphologically the tumours appeared to have been derived from type II cells. Repair of the O^6MG adduct also differed considerably among the pulmonary cell types of the rat and appeared to correlate with O^6MGMT activity, which was at least 50% less in Clara cells than in macrophages or type II cells (Belinsky, Devereux and Anderson, 1990). NNK treatment resulted in depletion of O^6MGMT in Clara cells, type II cells and small cells, resulting in insufficient activity to remove the higher levels of adducts in the Clara cells (Belinsky, Devereux and Anderson, 1990). In contrast to the lung, the levels of O^6MG in liver declined, over a 4 week treatment period, presumably due to induction of O^6MGMT (Belinsky, Foley *et al*, 1990).

Studies in which cells or organ cultures from rats were incubated with NNK *in vitro*, have generally supported the *in vivo* findings (Belinsky, White *et al*, 1989; Devereux *et al* 1988; Murphy, Heilblum and Trushin, 1990). DNA binding did not appear to be directly related to the rate of metabolism of NNK but the data suggest that DNA pyridyloxobutylation may be involved in NNN induction of oesophageal tumours.

Recently, studies in mice treated with NNK demonstrated similar levels of O^6MG in Clara cells and type II cells of the lung (Belinsky, Devereux *et al*, 1992). The authors suggested that the GC to AT base-pair mutation resulting from the O^6-methylation of guanine, followed by proliferation of the type II cells in response to toxicity to the type I cells (type II cells are progenitors for type I cells), could account for the tumours originating in the type II cells rather than the Clara cells. Consistent with this hypothesis was the observation that a GC-AT mutation was detected in the K-*ras* gene in 85% of alveolar hyperplasias induced by NNK treatment in mice (Belinsky, Devereux *et al*, 1992). A different molecular mechanism is involved in development of pulmonary tumours by NNK in the rat, as activation of *ras* genes was not found in NNK-induced tumours in this species (Belinsky, Devereux and Anderson, 1990).

The relative importance of the DNA methylation and pyridyloxobutylation in NNK-induced mouse lung tumours has been investigated using the model compounds described previously (section 5.1.2.1) and also deuterium substituted analogues of NNK. AMMN produces the same methylating species as that formed following methylene hydroxylation of NNK, whereas NNKOAc results in pyridyl-oxobutylation of DNA. NNN was inactive in inducing lung tumours in mice, NNKOAc was only weakly tumorigenic and AMMN was very active in comparison to an equivalent dose of NNK, indicating that DNA methylation is the critical event in the initiation of mouse lung tumours by NNK (Peterson and Hecht,

1991). Administration of NNKOAc with AMMN enhanced the persistence of the O^6MG, possibly accounting for the observed synergism of these two compounds. Thus pyridyloxobutylation may increase the efficiency of DNA methylation by NNK. Studies with [4,4-D_2]-NNK, which would undergo methylene hydroxylation more slowly than NNK, and [CD_3]-NNK, which would be hydroxylated at the methyl carbon more slowly, also indicated that methylene hydroxylation, and hence DNA methylation was more important (Hecht, Morse *et al*, 1991). [CD_3]-NNK, which would result in lower levels of pyridyloxobutylation than NNK, induced slightly more lung adenomas per mouse than NNK and did not decrease O^6-methylation. The authors suggested that this indicated that pyridyloxobutylation could be a protective process. However, it is also possible that the O^6- CD_3 adduct produced from [CD_3]-NNK could be more persistent than the O^6-CH_3 adduct produced from NNK.

5.4 Modulation of genotoxicity

Repeated administration of aqueous extracts of snuff has been shown to decrease DNA methylation by NNK (Prokopczyk, Adams *et al*, 1987). No decrease in methylation was seen following pretreatment of rats with 0.002% nicotine in the drinking water leading the authors to conclude that the nicotine in the snuff was not responsible for the decrease in methylation. The ratio of O^6MG/7MG was decreased in livers of snuff pretreated rats, indicating that the snuff extract may have induced O^6MGMT leading to increased repair of adducts (Prokopczyk, Adams *et al*, 1987).

It is well established that the metabolism and carcinogenicity of TSNAs is inhibited by various phytochemical components of certain fruits and vegetables. The most commonly studied modulators of TSNA metabolism are the alkylaryl-isothiocyanates (found in cruciferous vegetables), particularly phenylethylisothiocyanate (PEITC), which are primary products of thioglucosidase-catalysed hydrolysis of glucosinolates and are generated naturally in saliva. Adminstration of PEITC to rats or mice *in vivo* resulted in inhibition of NNK metabolism in lung and oral tissue *in vitro* (Morse, Amin *et al*., 1989; Murphy, Heiblum, King *et al*, 1991), and in decreased DNA methylation and pyridyloxobutylation following a subsequent i.v. dose of NNK (Chung, Wang and Hecht, 1985; Morse, Wang *et al*, 1989). Inhibition occurred rapidly and did not result in a compensatory induction of the enzyme. *In vitro* studies have shown that PEITC inhibited the metabolism of NNK on a mole/mole basis suggesting that it is a very potent inhibitor.

Low concentrations of (+)-catechin, a flavonoid present in black and green tea, wine, chocolate and apple skin, inhibited α-hydroxylation of NNK (Castonguay, Pepin and Briere, 1991), whereas N-oxidation was only inhibited at concentrations in excess of 40μM. (Liu and Castonguay, 1991). Single strand breaks induced in rat hepatocytes *in vitro* by NNK were inhibited by (+)-catechin. Administration of catechin by gavage to rats, 1 hour before s.c. injection of NNK, also reduced hepatic DNA methylation (Liu and Castonguay, 1991).

Vitamin A has been shown to inhibit the α-hydroxylation of NNK in rat hepatocytes and to inhibit the SCEs induced by NNK in V79 cells (Alaoui-Jamali, Belanger *et al*, 1991).

Indole, L-tryptophan and indole-3-carbinol (all found in crucifrous vegetables) have been shown to be inducers of hepatic NNK demethylation to formaldehyde (Chung, Wang and Hecht, 1985), and resulted in enhanced hepatic DNA methylation (Morse, LaGreca *et al*, 1990). In contrast indole-3-carbinol decreased the formation of O^6MG in lung, presumably due to the increased hepatic clearance of NNK. Diallyl sulphide, a component of garlic oil, has also been shown to inhibit metabolism of NNK and also to inhibit its tumourigenesis (Hong, Wang *et al*, 1992).

5.5 Comment

In its intact form, smokeless tobacco is unsuitable for mutagenicity testing hence the use of extracts which, in theory at least, would contain putative carcinogens. Positive results were obtained when aqueous extracts were tested in bacterial mutagenicity tests and when organic or aqueous extracts were tested against cultures of human cells *in vitro*. The significance of these results is not clear. It is generally recognised that extraction produces samples which are unrepresentative of the original material especially with respect to mutation-inhibiting substances.

TSNAs are mutagenic both *in vitro* and *in vivo*. NNK and NNN are mutagenic to *Salmonella typhimurium* TA100 in the presence of a metabolic activation system, indicating that they induce point mutations. The principal mutation recovered from the *lacI* gene of NNK-treated *Escherichia coli* were of the GC to AT transition, confirming the induction of point mutations and consistent with the major product of NNK adductions being O^6MG. Other mutations were also detected, in accord with the complex pathways of activation of NNK, and a possible role of DNA pyridyloxobutylation. NNK and NNN are mutagenic to various mammalian cells *in vitro* as detected by mutations, chromosomal aberations and DNA damage, generally at concentrations in excess of 0.1mM. It has been demonstrated

that this mutagenic potential is expressed *in vivo* in the bone marrow of mice and in tracheal epithelial cells of rats.

Formation of DNA adducts has been demonstrated in liver, lung, nasal mucosa, oesophagus and kidney of rats following administration of NNK. Studies generally indicate that pyridyloxobutylation of DNA is of secondary importance to DNA methylation. DNA methylation was greater in the respiratory mucosa than in the olfactory mucosa. This is in contrast to the greater sensitivity of the olfactory mucosa to NNK-induced tumours, indicating that tumourigenicity is not solely determined by adduct formation. Similarly levels of DNA methylation in different populations of lung cells do not correlate with NNK-induced tumours originating in the type II cells. The cell specificity was attributed to the combined effects of cytotoxicity and DNA alkylation.

The inhibition of metabolism of TSNAs by dietary constituents (especially phytochemical compounds) correlates with inhibition of mutagenic potency and formation of DNA adducts. Induction of oxidative metabolism is generally associated with a decrease in adduct formation in extrahepatic tissues due to increased hepatic clearance of the TSNAs. In addition, repeated administration of aqueous snuff extract, which induced oxidative metabolism of TSNAs, decreased adduct formation, apparently due to induction of repair enzymes increasing the rate of repair of the methyl adducts. These factors combine to decrease the potential mutagenic effects on the TSNAs within the smokeless tobacco.

Thus, it has been demonstrated that NNK and NNN can interact with DNA and produce mutations in a number of different mammalian cell types, indicating that they have the potential to be carcinogenic. However, it does not follow that these nitrosamines, within smokeless tobacco, will induce tumours of the oral cavity. The aromatic amino/nitro class of chemical molecules are known to contain a high proportion of experimental carcinogens and mutagens, but has also been shown to be responsible for a disproportionately high number of false-positive mutagenicity responses in the U.S. National Toxicology Program (NTP) (Ashby and Tennant, 1991). It appears, therefore, that mutagenicity assays are overly sensitive to this class of compound.

6 STUDIES ON THE INTERACTION OF VIRUSES AND SMOKELESS TOBACCO

6.1 Introduction

There are at least two families of viruses which have been implicated in mammalian neoplasia - the herpes viruses and the human papilloma viruses.

Human papilloma viruses (HPV) are a group of small DNA viruses that induce benign skin lesions, including squamous warts and papillomas in man (Munger *et al*, 1992). A subgroup of about twenty viruses have been associated with proliferative lesions in the anogenital tract. This subgroup has been further divided into two groups: the "low-risk" HPVs (HPV-6 and HPV-11) associated with benign proliferative lesions such as condyloma acuminata and the "high risk" HPVs such as HPV-16 and HPV-18 which are associated with lesions that can progress to cancer. For example 85% of cervical carcinomas contain HPV DNA sequences some of which belong to the "high risk" HPV DNA (Hausen and Schneider, 1987; Riou *et al*, 1990).

A number of HPVs have been associated with oral proliferative lesions in man and are thought to be one of the causative factors that lead to the development of malignancy. Some HPVs have been associated with oral leukoplakia and with keratoses of the type found in users of smokeless tobacco (Greer *et al*, 1987). Recently, Watts *et al* (1991) have shown that approximately 60% carcinomas from the oral region (floor of mouth, tongue, pharynx and larynx) were positive for DNA from a variety of HPV viruses including the high risk HPV-16 or HPV-18.

It would thus appear that HPV may be implicated in the development of oral carcinoma in man. However, there does not appear to be any evidence from work on experimental animals to identify the extent to which HPVs are involved in this neoplastic process and whether external agents can influence their activity in this respect. It is an obvious area for future research.

Herpes viruses form a large group which infect most mammalian species. There are four types which have been implicated in human disease: Epstein Barr Virus (EBV), cytomegalovirus (CMV), herpes simplex virus (HSV) and varicella-zoster virus (VZV) (Shillitoe and Silverman, 1979). The Epstein-Barr virus is almost certainly the cause of Burkitt's lymphoma (de The *et al*, 1978) and cytomegalovirus is thought to cause Kaposi sarcoma (Giraldo *et al*, 1978). HSV can cause acute and recurrent infections in the oral or genital epithelia in man and has been linked with cancer development at both of these sites (Rawls *et al*, 1969; Shillitoe and Silverman, 1979). HSVs are divided into two principal types, HSV-1 and HSV-2, according to the production of small (HSV-1) or large (HSV-2) vesicles when injected into the chorioallantoic membrane of the chick embryo. HSV-1 is generally isolated from the oral cavity epithelium and HSV-2 is primarily found in the genital regions but the division is not absolute since either virus may be isolated from these anatomical sites (Shillitoe and Silverman, 1979). HSV-1 virus is ubiquitous and approximately 70% of the adult population in the USA and Western Europe is seropositive for this virus (Larsson *et al*, 1989).

According to Shillitoe and Silverman (1979) the course of the infection by HSV-1 is often divided into three stages. During the primary stage vesicular lesions appear and are localised to the mucous membranes but the virus travels back along the sensory nerves towards the regional sensory ganglion where it may remain latent for several years. Subsequent herpetic infections are not due to exogenous re-infection with HSV but to the release of virus from latency followed by its passage along the sensory nerves to the skin. Reactivation of the virus is not always accompanied by epithelial cytolysis indicating that the epithelial cells can be repeatedly exposed to HSV without being killed (Shillitoe and Silverman, 1979).

A substantial number of reports have associated HSV-1 with leukoplakias, epithelial dysplasia and cancer of the oral cavity in man (Shillitoe and Silverman, 1979) and experimental investigations were conducted to define the conditions under which the virus can induce tumours. In early *in vitro* studies Duff and Rapp (1971) showed that if the cytolytic properties of the virus were inhibited by exposure to UV-light, the virus became capable of transforming hamster cells in culture. These cells grew into carcinomas if they were re-introduced into the original host. Subsequently, it was shown that cultured cells from a variety of species can be transformed under these conditions both by HSV-1 and HSV-2 (Rapp and Shillitoe, 1978).

Furthermore, Park, Sapp *et al* (1986) drew attention to the possibility that a number of agents other than UV light may attenuate the cytolytic properties of HSV in the course of the early infection or during the reactivation phase thus enabling the emergence of the carcinogenic properties of the virus. This comment would apply particularly to snuff because of the long time it is retained in the mouth.

The experimental evidence for the involvement of HSVs is reviewed in the following sections.

6.2 *In vitro* studies

One of the earliest and most comprehensive studies on the effect of tobacco extracts on HSV replication *in vitro* was conducted by Hirsch, Johansson and Vahlene *et al* (1984). Aqueous extracts were prepared from three different brands of snuff and their toxicity was first established by exposing Green Monkey Kidney (GMK) cells to various dilutions of the extract. It was found that the undiluted extracts produced a slight toxic effect on the cells and reduced the infectivity of the virus. At lower concentrations there were no such effects and a dose- related decrease in plaque formation, indicative of inhibition of virus replication, was observed. Further tests revealed that the extracts blocked HSV reproduction at an early

stage, probably at the stage of viral DNA replication. Additional tests on these three brands revealed that nicotine and TSNAs in the amounts (concentration not given) found in the three tested brands could inhibit HSV replication.

Stich, Li *et al* (1987) also found that aqueous extracts of snuff reduced the growth of HSV-1 virus in Vero cells in a concentration-dependent manner, the higher the concentration of snuff extract, the greater the inhibition. Snuff extract also reduced significantly HSV-1 DNA synthesis in a concentration-dependent manner in Vero cells. At the higher concentration, the DNA synthesis of Vero cells themselves was reduced but at lower concentrations only the viral DNA synthesis was affected. Furthermore, HSV-1 was inactivated and its cell lysing activity was lost when it was incubated with snuff in cell free conditions.

Suk *et al* (1990) confirmed these findings. They found that an aqueous extract of snuff inhibited the replication of HSV-1 in Vero cell monolayers in a concentration-dependent manner. At higher concentrations the DNA replication of the Vero cells themselves was also inhibited. They further found that the production of proteins essential for the viral DNA synthesis was also inhibited suggesting that snuff extract inhibits HSV-1 DNA replication via the alteration of viral protein synthesis. But the inhibition was not total; a proportion of these proteins was continuously being produced even in the presence of the highest concentration of snuff extract indicating that concentrations of snuff extract which inhibit cell lysis by inactivating the cytolytic ability of the virus do not totally abolish the expression of viral genes. This is a situation which, according to Park, Akoto-Amanfu and Paik (1988), may increase the risk of cell transformation (oncogenic capacity) by the virus.

Later studies revealed that NNN, NNK and an aqueous extract of snuff (NNN or NNK up to 10µg/ml and snuff extract up to 5% of culture medium) had no effect. This is thought to be due to an induction of DNA repair mechanisms by the carcinogens (Dokko *et al*, 1991). The authors thought that an increase of repair mechanisms might make them error-prone and result in DNA misrepair which might augment viral integration into the host cell.

Park, Dokko *et al* (1991) reported similar findings. They exposed NIH/3T3 cells to HSV-1, NNN or NNK and found that the transformation frequency (foci of transformed cells/10^6 cells) was, respectively, 12, 42 and 47. The addition of NNN at a range of concentrations (1-10 µg/ml) to the culture medium followed by UV-irradiated and HSV-1 inoculation increased the transformation frequency at all concentration levels by three-fold. Under the same conditions, addition of NNK at the same range of concentrations resulted in a four-fold increase in the frequency of transformation.

This *in vitro* work shows that TSNAs and aqueous snuff extract can inhibit the cytolic ability of the HSV-1 virus thus leading to cell transformation. They can also potentiate the rate of UV- induced cell transformation.

6.3 *In vivo* studies

6.3.1 Oncogenes

There is considerable evidence that mammalian cancer is the product of genomic instability. The critical target genes believed to be of primary importance in cancer production are oncogenes, tumour suppressor genes, metastasis genes and senescence genes. Oncogenes are activated by a number of factors such as viruses and chemical carcinogens. The effect of chronic snuff dipping on oncogene activation was studied by appropriate techniques in animals. Hamster buccal pouches were exposed to snuff twice daily, 5 days a week for 40 weeks and oncogene expression was determined by the viral probe oncogene v-erb-b. The results indicate that chronic simulated snuff dipping alone does not induce the amplification of known cellular proto-oncogenes c-erb-b in epithelial tissues of hamster buccal pouches. In the hamster cheek pouch cells infected with HSV-1 virus there was a slight increase in v-erb-b hybridisation to the cellular DNA indicating that c-erb-b may be amplified by snuff dipping and continuous infection with HSV-1 (Park, Akoto-Amatu and Paik, 1989). According to these authors, this oncogene along with other erb-b oncogenes have been well documented as "a causative factor" in various human tumours.

6.3.2 Pathology and virus replication

The role of snuff in the production of oral cavity tumours in animals infected with HSV-1 virus has been investigated in a number of studies after the demonstration by Hirsch, Svennerholm and Vahlne (1984) that it was possible to produce an acute HSV-1 infection in rats artificially, by scarification of the lips and by applying a culture of the virus on the scarified area. Recovery of the virus from the trigeminal ganglion was only possible in 10% of the infected animals so that the virus has to be repeatedly applied to the mucosa to maintain its presence. Park, Herbosa *et al* (1985) inoculated groups of male mice (BALB/c) with an HSV-1 virus propagated in Vero cell monolayers and studied the lesions which the inoculated virus produced in the lips. They also studied the effect of applying a water extract of snuff on the development of the viral lesions. The snuff extract was applied three times a day, five days a week for two months. Distilled water was applied in the same way to controls inoculated with the HSV-1 virus. Groups of mock-inoculated mice (scarified and swabbed with sterile culture medium) were also

included. The animals were observed every day for the 2-month duration of the experiment when all animals were killed.

It was visually observed that on the third post-inoculation day the inoculated area became erythematous and oedema appeared on the following day. Small vesicles appeared on the fifth day and ulceration occurred on the seventh day. Thereafter, the lesion began to heal and was completely healed by the fourteenth day after inoculation. Topical application of aqueous snuff extract reduced slightly the severity of the lesion. At the end of the experiment no lesions were observed in mice inoculated with the virus only or in mock-inoculated mice treated topically with distilled water or snuff extract. Histologically, a mild reaction was found in the lips of these mock-inoculated mice consisting of slight hyperkeratosis, inflammatory cell infiltration and acanthosis. In HSV-1 infected mice, the hyperkeratosis, acanthosis and inflammatory cell infiltration were only slightly more pronounced than in the previous groups, but a severe reaction was observed in mice infected with HSV-1 virus and treated topically with aqueous extract of snuff. In addition epithelial "atypia" were found in this group.

The demonstration that application of an aqueous extract of snuff to the lips of mice inoculated by the HSV-1 virus caused a much more severe reaction than either applied on their own led to an investigation to determine whether this severe reaction implied an activation of the virus (Park, Herbosa and Sapp, 1987). Groups of thirty BALB/c mice were inoculated with HSV-1 cultures on their upper lips and then painted with either distilled water or an aqueous extract of snuff three times daily, five days a week for two or three months. A group of "mock" inoculated animals (scarified lips without virus inoculation) was also included. At the end of the experiment, all mice were killed and trigeminal ganglia were removed and examined for the presence of virus. Swabs for viral cultures were taken on the seventh and thirteenth post- inoculation days.

The clinical features of the disease were closely similar to those described in the previous paper (Park, Herbosa *et al*, 1985). Viral cultures taken from the lips of HSV-1 inoculated mice of all treatment groups were positive for HSV-1 on the seventh post inoculation day but negative on the thirteenth day. In mock inoculated mice cultures were negative on both days. At the termination of the experiment all HSV-1 inoculated mice had established a latent infection (in the trigeminal ganglion) with this virus but none of the mock inoculated ones did so. Topical application of distilled water or snuff extract to the site of virus inoculation in the upper lip did not induce the shedding of the virus at the site of inoculation. Thus, the severe reaction reported by Park, Herbosa *et al* (1985) at the site of virus inoculation followed by the application of snuff extract may not signify activation of HSV-1 virus.

Rats infected by HSV-1 virus were used by Hirsch, Johansson and Vahlne (1984) to investigate the possible role of snuff applied regularly to the oral mucosa of infected rats in the development of oral carcinomas. Employing the "lip-canal" model (see Section 2.4), the following groups of ten rats were studied:-

i) untreated;

ii) treated with snuff insertion daily for eighteen months;

iii) infected with HSV-1 virus without further treatment;

iv) infected with HSV-1 virus and treated with snuff.

The results of this study did not indicate any carcinogenic response to either HSV-1 alone or snuff alone. However in the group treated with both HSV-1 and snuff there were two carcinomas in the oral cavity. The non-neoplastic pathological changes in the oral cavity followed the same course as those noted earlier (see Section 2.4). Apart from the lip canal there was no significant difference between any of the groups in the incidence of tumours. In this investigation, virus was cultured regularly from the oral cavity of inoculated rats while no virus was isolated from the uninfected animals.

Larsson, Johansson et al (1989) used the rat lip canal model to compare the actions of snuff as a promoter to virally "initiated" cells with its actions on chemically "initiated" cells (see section 2.5).

In the virus part of the study 55 Sprague-Dawley rats were divided into four groups and all had surgery to create an artificial lip canal. Two groups (I and III - see Table 27) were inoculated with HSV-1 once monthly for the duration of the study. Group III were then treated with 200mg Swedish snuff in the test canal twice daily five days a week for the duration of the study allowing an average exposure of twelve hours per day. Two further groups (controls) were included. One (Group VI) was left untreated after construction of the lip canal and the other (Group II) received snuff in the same way as Group III but no other treatment. The study lasted for 30 months except for those rats which were killed earlier for humane reasons.

Squamous cell carcinomas of the lip canal or close to its orifice were found in one rat in each of Groups I and II. There were no tumours of the oral cavity in Group III. Other tumours occurred systemically. They were mostly single tumours and appeared to be unrelated to treatment.

The number of tumours of the oral cavity observed in this study is too small to be conclusive and there is no indication of any interaction between exposure to snuff and exposure to HSV-1 in this study.

In a study on Golden Syrian hamsters, Park, Sapp and Herbosa *et al*, (1986) divided one hundred and twenty-five males into seven groups of fifteen to twenty animals per group as follows:-

i) No treatment;

ii) Mock inoculation;

iii) Mock inoculation plus simulated snuff dipping;

iv) HSV-1 inoculation;

v) HSV-2 inoculation;

vi) HSV-1 inoculation plus simulated snuff dipping;

vii) HSV-2 inoculation and simulated snuff dipping.

The viral inoculations were repeated once every four weeks for twenty-four weeks. Approximately 150mg of snuff was placed in each pouch twice a day for six months when the experiment was terminated. Ten out of twenty and 11/20 hamsters from Groups (vi) and (vii) respectively had developed invasive squamous cell carcinoma of the cheek pouch. No tumours developed in any one of the other groups.

In a subsequent experiment Park, Min et al (1993) investigated the interaction between HSV-1 and NNN, NNK or BaP in Syrian Hamsters. Groups of 20 hamsters were inoculated repeatedly (presumably every 4 weeks) with 100 µl culture medium or with the same amount of culture medium containing HSV-1 into the right buccal pouch. The left pouch was left untreated. The site of inoculation was then treated with topical application of 1% NNN, NNK or with BaP, each dissolved in mineral oil, or with mineral oil alone. The topical applications were carried out three times a week, for a period of 15 weeks for BaP and 20 weeks for mineral oil, NNN or NNK. The experiment was terminated 30 weeks after the first inoculation. No tumours were observed in the pouches of any of the groups except the two groups treated with BaP. There was a low incidence of pouch tumours in the group treated with culture medium plus BaP and a higher incidence in the group treated with HSV-1 plus BaP (see Table 28).

6.4 Comment

There is little doubt that HSV-1 infected cells *in vitro* can be transformed into potentially malignant cells by agents that suppress the cytolytic effect of the virus such as UV radiation and chemical carcinogens. This suppression is thought to be achieved by interference with the cellular synthesis of proteins essential for the growth of the virus while leaving intact the expression of the viral genome.

In vivo studies do not, however, present such a clear picture. A carcinogenic effect was produced when hamster cheek pouches, infected with HSV-1 virus, were exposed to snuff but this experiment would have been much more convincing if it had lasted more than six months. If the animals had been kept longer and the differences in tumour incidence had persisted there would have been a clear indication of an interaction between HSV-1 and snuff.

Some support for the role of snuff in activating the potential carcinogenic properties for HSV-1 was provided by Hirsch, Johansson and Vahlne (1984) who reported two carcinomas in the oral region of rats infected by HSV-1 virus and treated with snuff. The number of tumours induced is, however, too small to be convincing and a subsequent experiment on similar lines by Larsson, Johansson *et al* (1989) was negative with respect to interaction between HSV-1 and snuff. No tumours were produced when NNK and NNN were applied topically to the cheek pouches of hamsters inoculated with HSV-1. BaP, under the same conditions, produced a low incidence of tumours when applied alone and a higher incidence when applied to virus inoculated hamsters.

The reason for these inconclusive results is not clear but is in keeping with the demonstration that snuff or its aqueous extract does not activate a latent virus infection suggesting that it is not irritant enough and does not contain enough carcinogenic TSNAs to do so. It is well recognised that trauma such as epilation, cellophane-tape stripping and topical application of dry ice (Park, Herbosa and Sapp, 1987) can activate a latent HSV-1 virus infection but these operations are likely to be much more traumatic than the application of snuff or snuff extract.

It is noteworthy that in the *in vivo* experiments mentioned in this section, snuff or snuff extract on their own, produced only a very low incidence of tumours, while NNK and NNN applied on their own were negative. The investigation showing that snuff does not activate oncogenes is in keeping with the absence of carcinogenicity of snuff.

7 AN OVERVIEW

The potential carcinogenicity of smokeless tobacco was investigated in laboratory animals presumably because of anecdotal accounts and epidemiological studies claiming an association between oral cancer and smokeless tobacco.

Most of the early experimental studies were conducted on the cheek pouch of hamsters because the cheek pouch represented an easily accessible area of buccal epithelium which could permit long-term

application of smokeless tobacco (see section 2.1). The methods employed in these studies varied from a single filling, which was left in place till the termination of the study, to removal and replacement of smokeless tobacco on a daily basis. The former method can be criticised on the basis that any water-soluble carcinogens present may be eluted within a short space of time after insertion so that no further contact of carcinogens with the epithelium of the cheek occurs. This method, however, allows carcinogens which are sparingly soluble in water to be eluted gradually by the saliva and to come into continuous contact with the cheek pouch epithelium. Frequent replacement of smokeless tobacco on the other hand, has the advantage of ensuring prolonged contact with the pouch mucosa by carcinogens that elute quickly. Thus both methods are useful for investigating the carcinogenicity of smokeless tobacco.

Whichever method was adopted, results of these long-term studies in the hamster cheek pouch, did not demonstrate a carcinogenic effect. In contrast, the introduction of various polycyclic aromatic hydrocarbon carcinogens in the hamster cheek pouch resulted in a high incidence of carcinomas even though the application was limited to a few weeks. This shows that the hamster cheek pouch is capable of developing carcinomas when exposed to carcinogens and that the negative results with smokeless tobacco were not due to an insensitivity of the cheek pouch epithelium to carcinogens.

It is interesting to observe that there was a mild to moderate hyperplasia (an increase in the number of cells) in the hamster cheek pouch which was accompanied by a mild to moderate inflammatory cell infiltration whether the application of smokeless tobacco was repeated or was carried out once only. No metaplasia (change in cell morphology) or fibrosis was ever reported.

Another method of obtaining long-term exposure of the buccal epithelium to smokeless tobacco is the use of a surgically-constructed lip canal in the rat. In contrast to the results with the hamster cheek pouch repeated insertion of smokeless tobacco in the lip canal of rats provoked a marked epithelial as well as a marked connective tissue reaction which intensified with the progress of the experiment. As discussed in Comments, Section 2.4, severe reactions of this sort are liable to produce tumours even in the absence of any chemical carcinogens.

The mildness of the reaction produced in the hamster cheek pouch suggests that smokeless tobacco itself is only a mild irritant so that some other factor must be responsible for the severity of the reaction observed in the lip canal. As pointed out in Comments, Section 2.4, the rubbing of the tissues constituting the lip canal by the implements used to clean out and refill it with smokeless tobacco must account for the considerable tissue damage observed. When one takes into account that the process is carried out twice daily it becomes evident that mechanical injury must have played a major role in

provoking the severe epithelial and connective tissue reactions observed and hence also in the production of the tumours observed. It would appear likely that in the confined space of the rat oral cavity the manipulations involved in filling the lip canal twice daily may have produced sufficient damage in the mucosal epithelium of the tongue and hard palate to produce the few tumours reported in this region.

In some of the studies on the lip canal, a low incidence of forestomach tumours was reported in rats treated by smokeless tobacco alone. The significance of the induction of forestomach tumours in rats and mice has been intensively studied and it has been shown that chronic damage to the epithelial cells will lead to the production of both benign and malignant tumours (Grasso *et al*, 1991). Thus the appearance of a few forestomach tumours in rats do not indicate that smokeless tobacco has any carcinogenic potency.

In long-term dietary studies on mice, rats and hamsters there was no evidence that smokeless tobacco possessed any carcinogenic potential. In the hamster study, which involved 2 years exposure, the negative control substance, cellulose, produced a low incidence of forestomach tumours.

Only one relevant topical application study with smokeless tobacco has been found. An aqueous extract of snuff was applied to the mucosa of the oral cavity for 2 years and no tumours were observed thus underlining the fact that smokeless tobacco is not carcinogenic in experimental animals.

Analytical studies revealed the presence of a number of nitrosamines in smokeless tobacco. Many of these are present in other products that come into contact with man but six non-volatile nitrosamines were only found in tobacco and were called "Tobacco-Specific Nitrosamines" (TSNAs). Several experiments were conducted to assess the carcinogenicity of TSNAs and two (NNN and NNK) have been found to be alkylating agents and to possess both mutagenic and carcinogenic properties. The other four were either non-carcinogenic or equivocally so. Both NNN and NNK produced tumours when administered by the oral or parenteral route in hamsters, mice and rats. The principal target organ by both routes in mice is the lung. In rats the nasal cavities appear to be the main region affected by both nitrosamines but NNN in addition produced an incidence of tumours in the oesophagus; in hamsters the most commonly affected site is the tracheal epithelium as well as lungs and nasal cavity. The liver was affected in all three species but at a relatively low level. Where more than one dose level was used, the incidence was dose related and both benign and malignant tumours were produced. A clear carcinogenic response was produced even when the respective nitrosamine was given for a limited period only. Thus NNK and NNN behave as systemic carcinogens, producing tumours away from the site of administration. The oral cavity does not appear to be a target organ of either NNN or NNK. Topical

application of either nitrosamine on its own to the tongue epithelium of mice did not result in the formation of oral tumours, but the application of a mixture of the two substances to the oral mucosa of the rat resulted in an incidence of benign tumours which was statistically significant.

Because oesophageal tumours were produced when NNN was administered in drinking water it could be claimed that it was a locally-acting carcinogen. This interpretation is invalid because parenteral administration of NNN also produced oesophageal tumours.

Several studies have been conducted to elucidate the metabolism of NNN and NNK and it would appear that the two nitrosamines are metabolised by the P450 family of enzymes. Both *in vitro* and *in vivo* studies have shown that the metabolic pathways in the tissues that are known to be 'targets' for tumour production by these nitrosamines do not differ substantially from the pathways found in tissues that are unresponsive to tumour production. Nevertheless marked quantitative differences have been observed which might explain differences in organ specifity of the tumorigenic response. Thus NNK and NNN are metabolised via the same pathways by the oesophageal mucosa of the rat but NNN, which produces tumours in this tissue, is metabolised much faster (2-10 times dependent on dose) than NNK. Quantitative differences of this sort were observed in certain selected organs.

An interesting and important feature of the metabolism studies is the number of agents, mainly products found in food, which can slow down or inhibit the metabolism of these nitrosamines. Smokeless tobacco itself would appear to act as an inhibitory agent *in vivo*. Whereas a mixture of NNK and NNN produced a few benign tumours when applied to the oral mucosa of rats, the same mixture added to smokeless tobacco produced a much lower tumour incidence. When mentioning TSNAs in smokeless tobacco it is important to bear in mind the very low concentrations at which they are present and the low level of exposure to smokeless tobacco users (e.g. Tricker and Preussman, 1991). Other nitrosamines, mainly dimethylamine and diethylamine, occur in some nitrate/nitrite preserved food at about the same concentrations as in smokeless tobacco. So far no reliable evidence has been produced which indicates that these levels of nitrosamines in food produce cancer in man. Thus the mere detection of carcinogenic nitrosamines in smokeless tobacco does not mean that smokeless tobacco is carcinogenic. Indeed animal studies have shown that smokeless tobacco is not carcinogenic.

Mutagenicity tests were originally devised to screen materials for carcinogenicity testing because it was believed that interaction between DNA and the carcinogen was essential for tumour production. Long-term animal tests had shown smokeless tobacco to be non-carcinogenic before mutagenicity screening tests were generally available. Moreover, smokeless tobacco itself is difficult to use directly in the

standard tests and so extracts have been used. The results in the Salmonella/microsome test and in some mammalian cell systems, including human cell lines, have been positive. However, since these mutagenicity tests are generally recognised to be exquisitively sensitive, and since extraction causes artificial separation of the components of smokeless tobacco, the positive short-term tests results do not over-ride the absence of carcinogenicity in long-term animal studies.

A high incidence of oral cancer was observed when snuff was applied to the oral cavity of hamsters which harboured an active infection with herpes simplex virus for the whole of the experimental period. No tumours were observed in hamsters or rats treated in the same way with smokeless tobacco but without an artificially-maintained continuing active virus infection.

Overall the experimental studies support the conclusion that smokeless tobacco has not been shown to be carcinogenic in the oral cavity of experimental animals.

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TABLE 1 **Tumour incidence [a] in male F344 rats [b] after the application of snuff in the lip canal. See page 12 of text. From Hecht, Rivenson *et al*, 1986.**

Group	Number of rats	Lip Canal	Tongue	Hard Palate	Floor of mouth	Nasal Cavity	Stomach
Control	10	0	0	0	0	0	1
Snuff	32	1+(1)	0	1	0	0	1
Extracted Snuff	21	0	1	1	0	0	1
Enriched Snuff	32	0	0	0	1	(1)	2

Notes:- (a) Numbers in brackets indicate malignant tumours, all others were papillomas.
(b) Surviving to 116 weeks

TABLE 2 **Incidence of proliferative lesions in male Sprague Dawley rats (28 or 29 per group) after the application of NQO to the hard palate and snuff to lip canal. See page 14 of text. From Johansson, Hirsch *et al*, 1989.**

	Tumours					Squamous cell hyperplasia				
	I	II	III	IV	V	I	II	III	IV	V
Lip and lip canal	1+(1)	-	-	1	-	24	6	4	25	10
Hard Palate	2+(1)	-	2	4	-	18	7	7	14	2
Tongue	-	-	2+(2)	1+(1)	-	-	-	-	-	-
Nasal Cavity	1+(1)	-	-	1	-	-	-	-	-	-
Oesophagus	-	-	1	-	-	-	-	-	-	-
Forestomach	1	-	2	2	-	18	4	6	18	1
Lip (sarcomas)	(2)	-	-	(3)	-	-	-	-	-	-

Numbers in brackets indicate malignant tumours.

Group I - Snuff insertion for 104 weeks.
Group II - Painting hard palate with propylene glycol (PG) for 4 weeks.
Group III - Hard palate painted with NQO in PG for 4 weeks.
Group IV - As in Group III but with snuff inserted in lip canal for a further 104 weeks
Group V - Insertion of saline in cotton wool for 104 weeks.

TABLE 3 **Tumour incidence in male Sprague Dawley rats (38–42 per treated group with 30 controls) after the application to the lip canal of NQO or DMBA followed by snuff. See page 14 of text. From Johansson, Saidi *et al*, 1991.**

	Sarcomas						Squamous cell tumours					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Lip canal	-	(9)	(10)	(1)	(25)	(1)	-	(1)	2	1+(1)	2+(1)	-
Hard Palate	-	-	-	(1)	-	-	-	(2)	(3)	(6)	(5)	-
Buccal mucosa	-	-	-	-	-	-	-	-	-	(1)	-	-
Nasal Cavity	-	-	-	-	-	-	(1)	-	-	(1)	-	-
Forestomach	-	-	-	-	-	-	-	(2)	-	-	(1)	-

Numbers in brackets indicate malignant tumours.

Group I - DMBA plus cotton pellet.
Group II - DMBA plus snuff.
Group III - Snuff.
Group IV - NQO plus cotton pellet.
Group V - NQO plus snuff.
Group VI - Saline plus cotton pellet.

TABLE 4 **Survival and tumour incidence in male F344 rats treated by topical application to the oral mucosa of snuff extracts, or NNN and NNK. See page 7 of text. From Hecht, Rivenson *et al*, 1986.**

Group	Amount of Nitrosamines (µg/application) *NNN*	*NNK*	Survival (weeks)	Cheek	Hard Palate	Tongue	Lung
Water control	-		103±34	-	-	-	1
Snuff extract	6.6	1.4	108±24	-	-	-	-
Snuff extract & NNN and NNK	74	15	106±32	1	1	1	2
NNN and NNK	68	14	106±39	6	1	2	1+(4)

Numbers in brackets indicate malignant tumours.

TABLE 5 Tumour incidence in hamsters (50 of each strain per dose group) dosed orally. See page 8 of text. From Homburger *et al*, 1976.

	Cellulose[a]	Snuff[a]	MCA (5mg/dose)	Snuff[a] + MCA (0.5mg/dose)	Cellulose[a] +MCA 0.5mg/dose)
Forestomach	3+(4)	7+(1)	50+(20)	1+(2)	6+(7)
Glandular stomach	0	0	(19)	(8)	(6)
Small intestine	0	0	(8)	0	0
Large intestine	(1)	(1)	1+(51)	(1)	(2)

Notes:- (a) 20% in diet
(b) MCA = Methylcholanthrene
(c) Numbers in brackets indicate malignant tumours.

TABLE 6 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice[a] dosed intraperitoneally

Dose (mg/mouse) and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
1x22 (T)	Thrice weekly	25F (25F)	33	Hecht, Chen, Hirota *et al* 1978	The numbers of mice with lung adenomas (with the average number of lung adenomas per mouse) were:- NNK 20(2.61): Vehicle controls 5 (0.20).
1x22 (T)	Thrice weekly	25F (25F)	37	Castonguay, Lin *et al*, 1983	All surviving NNK-treated mice had lung tumours (average 38 per mouse) of which about half were carcinomas. 4 vehicle control mice had lung adenomas (0.2 per mouse) In addition 1 NNK-treated mouse had liver adenomas and carcinomas and another had a papilloma of the nasal cavity.
0.2x21 (S)	Thrice weekly	30F (30F)	37	Hecht, Abbaspour & Hoffmann, 1988	All NNK-treated mice had lung tumours (average 7.2 per mouse) compared with 12 control mice with lung tumours (average 0.5 per mouse)
0.2x20 (S)	Thrice weekly	25F (25F)	33	Rivenson, Djordjevic *et al*, 1989	All NNK-treated mice had lung tumours (average 16 per mouse) including 2 mice with lung adenocarcinomas. 5 vehicle control mice had lung tumours - all adenomas (average 0.2 per mouse).
0.5, 1 or 2 (S)	Once only	15F (15F)	up to 30	Hecht, Morse & Amin *et al* 1989	This study was designed to establish some parameters for a rapid tumourigenicity bio-assay. It was found that 3.5 months after dosing with NNK is probably the optimum time to obtain a significant increase in lung adenomas at any of the doses used. The tumour yield was significantly higher if mice were fed a semi-synthetic diet at the time of dosing even if they were later fed a standard diet.

TABLE 6 (continued) Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice[a] dosed intraperitoneally

Dose (mg/mouse) and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
0.2, 0.3, 0.4 0.5, 0.6, 1 or 2 (S)	Once only	20F(20F)	16	Peterson & Hecht, 1991	The percentage of mice with lung tumours and the multiplicity (lung tumours/mouse) ranged from 10% (0.1) in the controls; 21% (0.2) at 0.2mg/mouse; 30% (0.3) at 0.4mg/mouse and 95% (6.3) at 2mg/mouse. Between 0.4 and 2mg/mouse the increase in tumour multiplicity was linear with dose but below 0.4mg/mouse the dose/response appeared to be flatter. There was a similar break at 0.4mg/mouse in the dose-response relationship of the amount of lung DNA methylation measured as O^6 methylguanine 14 days after dosing. Parallel studies indicated that methylation of DNA was correlated with NNK-induced lung tumours but pyridyloxobutylation was not. However, the latter increased the tumourigenic effect of DNA methylation.
2 (S)	Once only	28F-39F (18F-29F)	16	Morse, Eklind Amin *et al*, 1989	This study was designed to investigate the influence of orally-dosed alkylarylisothiocyanate (ITCs) of varying alkyl chain length on the tumourigenicity of NNK. The ITCs were orally dosed at 5μ mol/mouse/day on 4 consecutive days before the NNK. The mice dosed with NNK but not ITCs all had lung adenomas (average 9.2/mouse). The ITCs with zero or one alkyl carbon (phenyl or benzyl ITC) had no effect on the tumourigenicity. A 2-alkyl carbon ITC (phenylethyl ITC) significantly reduced the multiplicity of lung adenomas. Three and 4-alkyl ITCs reduced both the incidence and multiplicity of lung adenomas.
2 (S)	Once only	20F-30F[c]	16	Morse, Hecht & Chung, 1990	This study extends the previous one to show that a dose of phenylethyl ITC of 25 μ mol/mouse/day reduces the incidence and multiplicity of lung adenomas to the same level as in the vehicle control group.
2 (S)	Once only	20F (20F)	16	Morse, Eklind, Hecht, Jordan *et al*, 1991	This study extends the previous one and shows that ITCs with increasing alkyl chain length up to at least C_6 are increasingly effective in inhibiting NNK tumourigenesis. The ITCs were dosed at 5, 1 or 0.2 μ mol/mouse/day and the C_6 (phenylhexyl) ITC reduced the multiplicity of lung adenomas by 85% at the lowest dose. ITCs containing a pyridyl instead of a phenyl ring were not effective inhibitors.

TABLE 6 (continued) Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice[a] dosed intraperitoneally

Dose (mg/mouse) and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
2 (S)	Once only	20[c]	16	Morse, Eklind Amin & Chung, 1992	Separate groups were orally dosed with phenylethyl ITC (5μmol/dose) either (as previously) on 4 consecutive days before NNK dosing or once only 2 hours before NNK dosing. The results were consistent with those of previous studies and showed that tumour inhibition by ITCs were due mostly, if not entirely to the final dose.
2 (S)	Once only	20F-24F (10F)	16	Morse, Reinhardt *et al* (1990)	This study used a similar protocol to the previous one except that the ITCs (with one or two alkyl carbons) were given in the diet and after dosing with NNK. There was little, if any inhibition of tumourigenesis.
2 (S)	Once only	20F-30F[c]	16	Morse, La Oreca *et al*, 1990	In this study Indole-3-carbinol, orally dosed at 25 or 125 μ mol/mouse/day for 4 days before dosing with NNK, reduced the multiplicity of lung adenomas by about 40% at both doses. There was little, if any, effect on the number of mice with adenomas.
1 or 2 (S)	Once only	30F (30F)	16	Hecht, Morse, Eklind and Chung, 1991	In this study NNK deuterated on the methylene carbon α- to the nitroso group produced significantly lower incidence and multiplicity of lung adenomas than ordinary NNK. Deuteration at the methyl carbon significantly increased the multiplicity of these tumours.

Notes:

(a) The mice were 6-8 weeks old at the start of the study;
(b) Where the publication gives dose in molar units this has been converted to mg (MW=207). Dosing vehicle: (S) indicates saline; (T) indicates trioctanoin;
(c) Vehicle control data taken from previous studies.

TABLE 7 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) after topical application on mice

Strain, sex and age at start of study	Dose (mg/mouse) and vehicle[a] x number of doses	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Sencar F 50-55 days old	0.03, 0.1 or 0.6 (A) all x10	Alternate days	29F or 30F (29F)	? 24	La Voie *et al*, 1987	NNK was applied to the shaved backs of the mice after which tetradecanoylphorbol acetate was applied twice weekly for 20 weeks. The tumour incidence (%) was:- HD (two studies): Skin tumours 79 and 59; Lung adenomas 45 and 63 MD: Skin tumours 24; Lung adenomas 10 LD: Skin tumours 10; Lung adenomas 10 Controls (two studies): Skin tumours 14 and 0; Lung adenomas 0 and 0 Skin tumours were statistically significant at HD and MD but lung tumours only at HD.
Swiss M and BALB/C M (both 8 weeks old)	1x22 (W)	Thrice weekly	30M (30M)	90	Padma, Lalitha *et al*, 1989 Padma, Amonkar and Bhide, 1991	NNK was applied to the ventral side of the tongue and the mice were given atropine in their drinking water to suppress salivation. The tumour incidence (% of those surviving more than 10 months) was:- Swiss: Lung adenoma 59; Forestomach papilloma 10; Hepatoma 7 BALB/C: Lung adenoma 36; Forestomach papilloma 36; Hepatoma 8 In an parallel study in a group of Swiss mice it was found that a vitamin A-reduced diet did not significantly affect the tumourigenicity of NNK. There were no oral tumours in any of the mice. None of the vehicle controls had any tumours. The 1991 publication reported that the NNK treatment reduced plasma vitamin A by >75% and liver vitamin A by 40% in both strains of mice.

Notes

(a) Where publication gives dose in molar units this has been converted to mg (MW=207). Vehicle:- (A) indicates acetone; (W) indicates water.

TABLE 8 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) by oral exposure in mice of two different strains[a]

Type of exposure	Strain of mouse and sex	Dose (mg/mouse)	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Drinking water	A/J F	Estimated total dose 3 or 9	Continuous for 7 weeks	19F-28F (24F)	23	Castonguay, Pepin and Stoner, 1991	The purpose of this study was to investigate the inhibition of the lung tumourigenicity of NNK by agents added to the diet before exposure to NNK and subsequently throughout the study. Of these agents β-carotene plus retinol and sodium selenite were not effective inhibitors. Ellagic acid, BHA and sulindac (an anti-inflammatory drug) did not affect the number of mice with lung adenomas but reduced the number of tumours per mouse by 52%, 88% and 52% respectively.
Gavage in water	Swiss M	1x22	5 days/week	17M(27M)	68-108	Bhide, Kulkarni *et al*, 1989	In the NNK-treated mice there were 14 lung tumours and 3 liver tumours. This compared with 2 lung tumours and one stomach papilloma in the controls. The tumour type was not stated.

Notes

(a) The mice were 6-7 weeks old at the start of the first study listed and 8 weeks old at the start of the second study.

TABLE 9 Transplacental carcinogenicity studies[a] of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in mice

Strain	Dose (mg/kg) ip to mothers x no of doses	Frequency of dosing (mothers) group (controls)	Number of offspring per dose group	Duration of study (weeks)	Observations and comments
A/J	100x3	Days 14,16 & 18 of gestation	32M:34F (48M:39F)	24	The lung tumour incidence (%) in the offspring was:- NNK / Controls Males 13 / 6 Females 24 / 3 In all groups there was an average of 1 lung tumour per tumour-bearing mouse.
$C3B6F_1$[b]	100x3	Days 14,16 & 18 of gestation	141M:124F (144M:144F)[c]	72	Promotion of NNK tumourigenicity was attempted by giving the offspring either sodium barbital in drinking water throughout the study or single oral doses of PCB on post-natal days 8 and 56. There was no significant difference in lung tumours between the NNK-exposed offspring and the controls either with or without attempted promotion. The total incidence of liver tumours in the NNK-exposed offspring was significantly higher than in the controls (40% v 17%) but this difference was slightly reduced by attempted promotion. The increased incidence of hepatocarcinoma in NNK-exposed males (9%) compared with controls (3%) and the difference persisted in the "promoted" groups.
NIH(S)	100x3	Days 15,17 & 19 of gestation	83M:63F (66M:64F)[d]	72	Promotion was attempted by giving single oral doses of PCB on post-natal day 56. Overall NNK appeared to have little effect on the incidence of lung tumours with or without "promotion". The liver tumour incidence was significantly higher in the NNK-exposed males (5%) than in the controls (0%) and this effect was accentuated by the PCB treatment (NNK 19% v controls 0%).

Notes
(a) The transplacental studies on NNK were all by Anderson, Hecht *et al*, 1989;
(b) Mothers were C3H/He strain;
(c) Four different groups of treated mice and controls;
(d) Two different groups of treated mice and controls.

TABLE 10 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Fischer 344 rats[a] dosed subcutaneously[b]

Dose and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
12mg/rat x 60	Thrice weekly	12M:12F (12M:12F)	52	Hecht,Chen,Ohmori & Hoffmann, 1980	The number of NNK-treated rats with tumours was:- lung adenoma 11; lung carcinoma 5 (all males), benign nasal 14; malignant nasal (mostly olfactory neurobastomas) 10; liver carcinoma 16; liver haemangiosarcoma 8. No tumours of these types were found in the vehicle controls.
30,10 or 3.3 mg/kg all x 60	Thrice weekly	HD 15M:15F MD 15M:15F LD 27M:27F (27M:27F)	120	Hoffmann, Rivenson *et al*, 1984	Dosing was discontinued for weeks 7-9 at HD because of toxicity. The number of rats with tumours (% of starting group numbers) was:-
					Apart from one benign oesophageal tumour in each of the MD and LD groups the range of other tumours in the treated groups was not significantly different from that in the controls.
1.1mg/kg x 60	Thrice weekly	30M(30M)	104	Hecht, Trushin *et al* 1986	The number of NNK-treated rats with tumours was:- lung adenomas 9; lung carcinoma 4; benign nasal 5; nasal carcinoma 1; benign liver 8; liver carcinoma 2. No tumours of these types were found in the vehicle controls.

	Lung adenoma	Lung carcinoma	Benign nasal	Malignant nasal	Malignant liver
HD	50	27	23	70	23
MD	17	50	50	33	17
LD	24	33	54	2	4

TABLE 10 (continued) Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Fischer 344 rats[a] dosed subcutaneously[b]

Dose and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
0.03-50 mg/kg all x 60	Thrice weekly	30M-62M (40M)	120	Belinsky, Foley *et al* 1990 and Belinsky, Devereux *et al* 1991	The number of rats with tumours (as % of starting numbers) was:-

Dose Rate	Benign liver	Malignant liver	Benign lung	Malignant lung	Benign nasal	Malignant nasal
0.03	0	0	5	2	0	0
0.1	2	0	5	5	0	2
0.3	0	0	8	5	0	0
1	3	3	40	13	0	0
10	10	0	37	37	63	17
50	39	44	16	71	74	44

Among the vehicle controls there was only one tumour of these types (a malignant lung tumour). Parallel studies indicated that tumours of the liver and nasal cavity only occurred at dose levels which caused cytotoxicity. No cytotoxicity was observed in the lung at dose levels which caused tumours.

Notes

(a) The rats were 7-10 weeks old at the start of the study;
(b) The dosing vehicle was trioctanoin;
(c) Where the publication gives the dose in molar units this has been converted to mg (MW=207).

TABLE 10 (continued) Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Fischer 344 rats[a] dosed subcutaneously[b]

Dose and vehicle[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
3.3 or 1.1 mg/kg both x 60	Thrice weekly	30M(30M)	104	Hecht, Lin *et al* 1987	NNK was compared with NNK deuterated at the methylene carbon α to the nitroso group or at the methyl carbon. The distribution of tumours in the rats dosed with undeuterated NNK was similar to that in previous studies at the same dose levels except that there were more reported benign hepatomas at the higher dose in the later study. The only effect of deuteration at either carbon on tumour incidence was to increase the number of malignant tumours of the nasal cavity.
1.8mg/kg x 60	Thrice weekly	40M(40M)	104	Morse,Hecht,Chung 1990 and Morse, Wang *et al* 1989	Two groups were similarly dosed with NNK but, while one was fed a standard diet throughout the study, the other was fed a diet containing 3μ mol/g phenylethylisothiocyanate (PEITC) for one week before and two weeks after dosing with NNK. The number of rats with tumours was:-

	Lung adenoma	Lung carcinoma	Benign nasal	Nasal Carcinoma	Benign Liver	Liver Carcinoma
NNK	8	24	8	3	12	3
NNK & PEITC	5	12	6	1	9	5

The authors concluded that PEITC reduced the incidence of lung tumours but not the incidence of tumours of the liver or nasal cavity.

TABLE 11 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[a] by topical application to the lips and oral cavity in male Fischer 344 rats[b]

Dose (µg/rat)[c] x number of doses	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
14 NNK plus 68 NNN x 1430 (mean)	Once or twice per day	30M(21M)	106 (mean)	Hecht,Rivenson *et al* 1986	Of the rats treated with NNN plus NNK 8 had oral papillomas and 5 lung tumours (1 adenoma and 4 adenocarcinomas). In a parallel group given the same doses of NNN plus NNK but with snuff extract in addition 3 rats had oral papillomas and 2 lung adenomas. A group given snuff extract only had no oral or lung tumours. In the vehicle controls there were no oral tumours and 1 lung adenoma. Other tumours were not significantly different between the treated rats and the controls.
930 NNK x 578	3 times for week 1 5 times/week for weeks 2-4 10 times/week for weeks 5-61	30M(30M)	61	Prokopczyk, Rivenson and Hoffmann, 1991	The number of NNK-treated rats with tumours was:- oral papilloma 1; lung adenoma 5; lung carcinoma 23; nasal papilloma 13; nasal carcinoma 2; liver adenoma 9; liver carcinoma 3. No tumours of these types were found in the controls.

Notes

(a) The dosing vehicle was water;
(b) The rats were 10 weeks old at the start of the study;
(c) Where the publication gives the dose in molar units these have been converted to µg (MW NNK = 207).

TABLE 12 Carcinogenicity studies on Fischer 344 rats[a] orally exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)

Type of exposure	Dose	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Gavage in corn oil/ethyl acetate vehicle	5mg/rat x 51	Twice weekly	12F(12F)	42 (median)	Lijinsky, Saavedra and Kovatch, 1991	The purpose of the study was to compare the organ specificity of the tumourigenicity of a range of nitrosamines. NNK was unusual in this range in that it did not cause tumours of the oesophagus. The number of NNK-treated rats with tumours was:- liver 10; lung 3; nasal mucosa 6.
Drinking water at concentrations of 0.5,1 or 5 ppm	Estimated total doses 6.9,15.6 and 63.5 mg/rat	Continuous throughout the study	HD 30M MD 80M LD 80M (80M)	105	Rivenson,Hoffmann *et al* 1988 Pour and Rivenson 1989	The number of NNK-treated rats with tumours was:-

	Lung Adenoma	Lung Carcinoma	Nasal (type not stated)	Pancreatic adenoma	Pancreatic carcinoma	Benign liver
HD	2	25	5	1	1	12
MD	16	4	2	8	1	11
LD	5	4	1	5	0	3
Control	3	3	0	1	0	6

The authors suggest that the reason for the comparatively low incidence of pancreatic adenomas at the HD is the lower survival in this group. The pancreatic tumours were of the exocrine pancreas mainly (75%) of acinar origin with the rest of ductal origin. However, one of the latter was shown to have mixed ductal-squamous-islet cell components.

Notes

(a) The rats were 7 or 8 weeks old at the start of the study.

TABLE 13 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) dosed subcutaneously[a] in Syrian golden hamsters[b]

Dose	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Group 1 - 10mg/hamster x 19 Group 2 - 2.5mg/hamster x 75	Thrice weekly	15M:15F (15M:15F)	65-70	Hoffmann and Castonguay *et al* 1981	The number of hamsters with tumours (as % of survivors) was:- Grp 1: Lung adenoma 50; Lung adeno-carcinoma 10; Nasal papilloma 0; Nasal carcinoma 10; Tracheal papilloma 0 Grp 2: Lung adenoma 60; Lung adeno-carcinoma 20; Nasal papilloma 5; Nasal carcinoma 50; Tracheal papilloma 35 No tumours of these types were found in the vehicle controls.
1, 3.3 or 10 mg/hamster	Once only	10M:10F (10M:10F)	78	Hecht, Adams *et al* 1983	Some groups were exposed to cigarette smoke but only those groups which were sham-exposed to smoke are noted here. The number of hamsters with tumours was:- HD: Lung adenoma 3; Lung carcinoma 1; Nasal papilloma 2; Olfactory neuroblastoma 1; Tracheal papilloma 0 MD: Lung adenoma 0; Lung carcinoma 2; Nasal papilloma 1; Olfactory neuroblastoma 0; Tracheal papilloma 0 LD: Lung adenoma 4; Lung carcinoma 0; Nasal papilloma 2; Olfactory neuroblastoma 0; Tracheal papilloma 1 No tumours of these types were found in the vehicle controls.
12.5mg/kg x 104 (max)	Twice weekly	15M (15M or 24M)	up to 52	Schuller, Witschi 1990	Groups similarly dosed with NNK were kept either in an atomosphere with 70% oxygen or in ambient air. The numbers of hamsters with lung tumours were similar in both groups but, whereas the hamsters kept in ambient air had adenomas and adeno-carcinomas, the lung tumours in hamsters kept in hyperoxic conditions were classified as neuroendocrine carcinomas. Other effects of hyperoxia were to reduce the tumour latency period from 16 weeks to 8 weeks and to reduce the survival time by about a third.

Notes

(a) The dosing vehicle was trioctanoin;

(b) Hamsters were 8-10 weeks old at the start of the studies.

TABLE 14 Carcinogenicity studies of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)[a] after topical application in Syrian golden hamsters[b]

Dose	Frequency of dosing	Number per dose group (controls)[c]	Duration of study (weeks)	Reference	Observations and comments
Group 1. 1mg NNK x 120	Thrice weekly	30-40 both sexes	90	Padma, Lalitha *et al* 1989	The NNK was applied to the cheek pouch and the hamsters were given atropine in the drinking water to suppress salivation. The incidence of tumours (as % of hamsters surviving more than 8 months) was:-
Group 2. 1mg NNK mixed with 6mg H_2O_2 x 120	Thrice weekly	30-40 both sexes	66		
Group 3. 1mg NNK x 10	Twice daily on consecutive days	30-40 both sexes	66		
Group 4 as Group 3 but followed by 6mg H_2O_2/ hamster x 120	NNK twice daily on consecutive days. H_2O_2 daily.	30-40 both sexes	66		

	Lung adenoma	Forestomach papilloma	Cheek pouch papilloma	Hepatoma
Group 1	8	27	4	4
Group 2	34	37	12	3
Group 3	0	0	0	0
Group 4	22	19	3	9

In the untreated control group 30 hamsters survived more than 8 months and in the group treated only with H_2O_2 26 survived. No tumours of the above types were found in either of these groups.

Notes

(a) The dosing vehicle was water;
(b) The hamsters were 8 weeks old at the start of the study;
(c) It appears that the control groups were about the same size as the treated groups.

TABLE 15 Transplacental carcinogenesis of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in Syrian golden hamsters[b]

Dose (mg/kg) i.p. to mothers[a]	Frequency of dosing (mothers)	Number of offspring per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Group 1. 50	Once only on gestation day 15	38M:35F (40M:42F)	Up to 75	Correa *et al* 1990	The number of tumour-bearing offspring from NNK-treated mothers ranged from 29% to 76% and was approximately related to the total dose received by the mothers. The tumours were predominantly of the respiratory tract and consisted of benign tumours of the lungs, larynx and trachea, and adenocarcinomas of the olfactory epithelium of the nasal cavity. Other tumours in these offspring were in the adrenals, pancreas and liver. No tumours of these types were seen in the vehicle controls.
Group 2. 100	Once only on gestation day 15	36M:36F	Up to 75		
Group 3. 50 x 3	Gestation days 13, 14 & 15	39M:40F (43M:40F)	Up to 75		
Group 4. 200	Once only on gestation day 15	25M:41F	Up to 75		
Group 5. 100 x 3	Gestation days 13, 14 & 15	40M:37F			

Notes

(a) The dosing vehicle was trioctanoin;
(b) There were 5 mothers per dose group and for each of two control groups.

TABLE 16 Carcinogenicity studies of N-nitrosonornicotine (NNN) in A/J female mice[a] dosed intraperitoneally

Dose[b] (mg/mouse) x no of doses	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
1 x 22 (S)	Thrice weekly	25F(25F)	33	Hecht,Chen,Hirota *et al* (1978)	The number of mice with lung adenomas (with the average number of lung adenomas per mouse) were:- NNN 16 (1.74): Vehicle controls 3 (0.24). One NNN-treated mouse had an undifferentiated carcinoma of the salivary glands.
1 x 22 (T)	Thrice weekly	25F(25F)	33	Hecht,Chen,Hirota *et al* (1978)	The number of mice with lung tumours (with the average number of lung tumours per mouse) were:- NNN 12 with adenomas plus 1 with adenocarcinoma (0.87): Vehicle controls 5 adenomas (0.20). One NNN-treated mouse had an undifferentiated carcinoma of the salivary glands and another a malignant lymphoma.
1 x 22 (S)	Thrice weekly	24F(24F)	37	Castonguay,Lin *et al* 1983	16 NNN-treated mice had lung tumours (average 1.2 per mouse) including 10 with lung carcinomas. There were no tumours of other organs in this group. Seven control mice had lung tumours (average 0.4 per mouse) including 1 with a lung carcinoma.
0.8 x 21 (S)	Thrice weekly	30F(30F)	37	Hecht, Abbaspour and Hoffman, 1988	25 NNN-treated mice had lung tumours (average 1.8 per mouse) compared with 12 control mice with lung tumours (average 0.5 per mouse).

Notes

(a) The mice were 6-8 weeks old at the start of the study;
(b) Where the publication gives dose in molar units this has been converted to mg (MW = 177). Dosing vehicle: (S) indicates saline; (T) indicates trioctanoin.

TABLE 17 Carcinogenicity studies of N-nitrosonornicotine (NNN) in mice of two strains dosed intraperitoneally

Strain, sex & age at start of study	Vehicle	Dose[a]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Chester-Beatty M & F 6 weeks old	Arachis oil	2mg/ mouse x 41	Weekly	20M:20F (15M:15F)	41	Boyland *et al* 1964b	Seven of the 8 NNN-mice surviving for 8 months had multiple (invasive) lung adenomas and one a renal lympho-sarcoma. The only tumour in the vehicle controls was a single small pulmonary adenoma.
A/HE F age not stated	Saline	0.9mg/ mouse x 24	Thrice weekly	35F(31F)	32	Hoffman, Hecht *et al* 1976	Twenty-eight of the NNN-treated mice had lung adenomas (average 1.4/mouse) compared with 12 control mice (average 0.45 lung adenomas/mouse).

Note

(a) Where the publication gives the dose in molar units this has been converted to mg (MW = 177).

TABLE 18 Carcinogenicity studies of N-nitrosonornicotine (NNN) after topical applications on mice[a] of various strains

Strain and sex	Vehicle	Dose[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Sencar F	Acetone	0.5mg/ mouse x 10	Alternate days	27F(28F)	? 24	La Voie *et al* 1987	NNN solution was applied to the backs of the mice followed by twice-weekly applications of tetradecanoylphorbol acetate for 20 weeks. Two of the treated mice had skin tumours (not statistically significant) and none had lung adenomas.
Swiss M or BALB/c M	Water	1mg/ mouse x 22 (Swiss only) or x 72 (both strains)	Thrice weekly	30M(30M)	90	Padma,Lalitha *et al* 1989. Padma, Amonkar and Bhide, 1991.	NNN was applied to the ventral side of the tongue and the mice were given atropine in their drinking water to suppress salivation. The tumour incidence (% of those surviving more than 10 months) was:-

	Lung adenoma	Forestomach papilloma	Hepatoma
Swiss (x22)	37	26	0
Swiss (x72)	42	19	8
BALB/c (x72)	26	19	15

In a parallel study in a group of Swiss mice it was found that a vitamin A-reduced diet did not significantly affect the tumourigenicity of NNN. There were no oral tumours in any of the mice. None of the vehicle controls had any tumours.

The 1991 publication reported that the NNN reduced plasma vitamin A but not liver vitamin A in both strains of mice.

Notes

(a) The mice were 7-8 weeks old at the start of the study.

(b) Where the publication gives the dose in molar units this has been converted to mg (MW = 177);

TABLE 19 Carcinogenicity studies of N-nitrosonornicotine (NNN) in Fischer 344 rats[a] dosed subcutaneously

Dose and vehicle[b] x no of doses	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
10mg/rat x 60 (T)	Thrice weekly	12M:12F (12M:12F)	52	Hecht,Chen,Ohmori & Hoffmann, 1980	Apart from one (benign) liver tumour, the only compound-related tumours were in the nasal cavity. These consisted of olfactory neurobastomas (15 rats), rhabdomysarcomas (2 rats) and papillomas (3 rats). No tumours of these types were found in the vehicle controls.
27, 9 or 3 mg/kg all x 60(T)	Thrice weekly	HD 15M:15F MD 15M:15F LD 27M:27 (27M:27F)	120	Hoffmann, Rivenson *et al*, 1984	The numbers of rats with tumours (% of starting group numbers) was:-

	Lung adenoma	Lung carcinoma	Benign nasal	Malignant nasal	Benign oesophageal	Benign bladder
HD	3	0	0	90	23	3
MD	20	0	23	43	23	3
LD	7	2	43	7	4	4

No tumours of these types were found in the vehicle controls and the range of other tumours in the treated groups was not significantly different from that in the controls.

10mg/kg x 66 (S)	Thrice weekly	30M(26M)	98	Castonguay, Rivenson *et al*, 1984	Parallel groups were given ethanol (6% w/v) in a liquid diet 4 weeks prior to and during the dosing with NNN. The number of rats with tumours was:-

	Benign nasal	Malignant nasal	Benign oesophageal	Oesophageal carcinoma	Benign tongue
NNN	4	20	0	0	0
NNN & ethanol	2	20	2	1	1

No tumours of these types were found in either the vehicle only control group or the vehicle plus ethanol control group. There was no indication that NNN increased the incidence of other tumour types.

Notes

(a) The rats were 7 weeks old at the start of the first study listed here, 9 weeks old at the start of the second and 13 weeks old at the start of the third.

(b) Where the publication gives the dose in molar units this has been converted to mg (MW=177). Vehicle:- (T) indicates trioctanoin; (S) indicates saline.

TABLE 20 Carcinogenicity studies on Fischer 344 rats[a] orally exposed to N-nitrosonornicotine (NNN)

Type of exposure	Dose[b]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
Drinking water	Estimated total dose 630mg/rat	Continuous 5 days/week for 30 weeks	20M(20M)	47	Hoffmann,Rainerl *et al* 1975, and Hoffman,Hecht, Ornaff *et al* 1976.	The number of NNN-treated rats with tumours was:- Oesophageal papilloma 11; Oesophageal carcinoma 3; Pharyngeal papilloma 1; Nasal carcinoma 3. Apart from a metastasis from a primary oesophageal tumour no lung tumours were found. No tumours of these types occurred in the controls.
Drinking water	Estimated total dose M 637mg/rat F 584mg/rat	Continuous for 36 weeks	12M:12F (12M:12F)	43	Hecht,Young & Maeura 1983	The number of NNN-treated rats with tumours was:- Oesophageal papilloma 23; Oesophageal carcinomas 6; Benign nasal 15; Malignant nasal 15; Tracheal papilloma 1. No tumours of these types were found in the controls.
Liquid diet	Estimated total dose 177mg/rat	Continuous for 27 weeks	30M(26M)	98	Castonguay, Rivenson *et al*, 1984	Parallel groups were given ethanol (6% w/v) in their liquid diet 4 weeks prior to and during the administration of NNN. The number of NNN-treated rats with tumours was:- NNN: Oesophageal papilloma 16; Oesophageal carcinomas 9; Benign nasal 11; Malignant nasal 7; Tracheal papilloma 2. NNN & ethanol: Oesophageal papilloma 13; Oesophageal carcinomas 7; Benign nasal 20; Malignant nasal 6; Tracheal papilloma 3. No tumours of these types occurred in the controls and there was no indication that NNN increased the incidence of other tumour types.

Notes

(a) The rats were 6-8 weeks old at the start of the first two studies listed and 13 weeks old at the start of the third study;

(b) Where the publication gave the dose in molar units this has been converted to mg (MW=177).

TABLE 21 Carcinogenicity studies of N-nitrosonornicotine (NNN) in Syrian golden hamsters[a] exposed intraperitoneally or in their drinking water

Dose route	Vehicle	Dose (mg/hamster)[b]	Frequency of dosing	Number per dose group	Duration of study (weeks)	Reference	Observations and comments
Drinking water	..	Estimated total dose M:336 F:496	Continuous for 31 weeks	10M:10F (10M:10F)	96	Hecht, Young and Macura, 1983	The number of NNN-treated hamsters with tumours was:- Nasal papilloma 4; Tracheal papilloma 2; Liver angiosarcoma 1. No tumours of these types occurred in the vehicle controls
IP	Saline	2.4 or 4.7 both x 75	Thrice weekly	17M or 21M (19M or 21M)[c]	78	McCoy, Hecht *et al* 1981	Parallel groups were given ethanol (6% w/v) in a liquid diet 4 weeks prior to and during the NNN-dosing. The number of NNN-dosed hamsters with tumours (% of survivors) was:-

	Benign nasal	Malignant nasal	Tracheal papillomas	Liver nodules
LD plus control diet	0	5	19	0
LD plus ethanol	5	0	29	0
HD plus control diet	15	10	43	10
HD plus ethanol	15	5	33	10

No tumours of these types were found in the controls and there were no significant differences in the incidence of other tumours between the NNK-treated hamsters and the vehicle controls. The authors concluded that the tumourigenicity of NNN in the hamster was not influenced by ethanol. This was in contrast to the tumourigenicity of another nitrosamine (N-nitrosopyrrolidine) which was increased by ethanol.

Notes
(a) The age of the hamsters at the start of the studies ranged from 6-7 weeks old (first study listed); 13 weeks old (2nd study);
(b) Where the dose is given in molar units this has been converted to mg (MW=177);
(c) In this study the numbers per dose group are given as % of those surviving the NNN-dosing.

TABLE 22 Carcinogenicity studies of N-nitrosonornicotine (NNN) in Syrian golden hamsters[a] dosed subcutaneously

Vehicle	Dose (mg/hamster)[b] x no of doses	Frequency of dosing	Number per dose group	Duration of study (weeks)	Reference	Observations and comments
Saline	5 x 75	Thrice weekly	10M:10F (10M:10F)	83	Hilfrich *et al*, 1977	The number of NNN-treated hamsters with tumours was:- benign tracheal, 12; nasal carcinoma, 1. None of the vehicle controls had tumours of these types.
Trioctanoin	Group 1: 8.6x19 Group 2: 2.2x75	Thrice weekly	15M:15F (15M:15F)	65-70	Hoffmann, Castonguay *et al*, 1981	The number of NNN-treated hamsters with tumours (% of survivors) was:- Lung adenoma / Tracheal papilloma Group 1: 4 / 18 Group 2: 6 / 6 None of the vehicle controls had tumours of these types.

Notes

(a) The age of the hamsters at the start of the studies was 8-10 weeks.
(b) Where the dose is given in molar units this has been converted to mg (MW=177).

TABLE 23 Carcinogenicity studies in mice[a] of tobacco-specific nitrosamines present in tobacco in minor amounts

Nitrosamine[b]	Strain of mouse	Dose route	Vehicle	Dose[c] (mg/mouse) x no of doses	Frequency of dosing	Number per dose group (controls)	Duration of study	Reference	Observations and comments
Iso-NNAC	A/J	IP	Saline	2 x 20	Thrice weekly	5F(5F)	33	Rivenson, Djordjevic *et al* 1989	No significant differences in tumour incidence between treated mice and vehicle controls.
Iso-NNAL	Sencar	Topical (dorsal skin)	Acetone	0.6x10	Alternate days	29F(29F)	724	La Voie *et al* 1987	Tumourigenicity was promoted by twice weekly applications of tetradecanoyl-phorbol acetate for 20 weeks. Although 7% of the Iso-NNAL-treated mice had skin tumours and there were none in the vehicle controls, this incidence was below the level of statistical significance. Neither group had lung tumours.

Notes

(a) The mice were 6-8 weeks old at the beginning of the studies;
(b) Iso-NNAC is 4-(methylnitrosamino)-4-(3-pyridyl)-butyric acid which, besides being a minor constituent of processed tobacco, may be a mammalian metabolite of cotinine. Iso-NNAL is 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol.
(c) Where the publication gives the dose in molar units this has been converted to mg (MW: Iso-NNAC = 223; Iso-NNAL = 209).

TABLE 24 Carcinogenicity studies of N-nitrosoanabasine (NAB) in rats[a] exposed in their drinking water

Strain of rat	Dose (mg/rat)	Duration of exposure	Number per dose group (controls)	Duration of study (weeks)	Reference	Observations and comments
F344	Estimated total dose 630	5 days/week for 30 weeks	25M(20M)	48	Hoffmann, Rainert *et al* 1975	The number of NAB-treated rats with tumours was:- Oesophageal papilloma 1; Pharyngeal papilloma 1. None of the control group had tumours of these types.
Chester-Beatty	Estimated dose 5/day	6 days/week throughout the study	16M:16F (16M:16F)	74	Boyland *et al* 1964a	25 of the NAB-treated rats had multiple oesophageal tumours of which 5 were malignant. No oesophageal tumours were found in the controls and there were no other differences in tumour incidence between the treated rats and historical controls.

Note

(a) The rats were 7 weeks old at the start of the studies.

TABLE 25 Carcinogenicity studies of metabolites of tobacco alkaloids or tobacco-specific nitrosamines in female A/J mice[a] dosed intraperitoneally

Metabolite[b]	Dose (mg/mouse) and vehicle[c] x no of doses	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks	Reference	Observations and comments
NNA (a metabolite of nicotine)	1 x 22(S)	Thrice weekly	25F(25F)	33	Hecht,Chen *et al* 1978	9 NNA-treated mice had lung adenomas (average 0.4 per mouse) compared with 3 vehicle control mice average 0.2 per mouse).
NNAL (a metabolite of NNK)	1 x 22 (T)	Thrice weekly	25F(25F)	37	Castonguay, Lin *et al* 1983	All NNAL-treated mice had lung tumours (average 26 per mouse) of which about a third were carcinomas. In addition there was one nasal cavity papilloma and one papilloma of the tongue in the treated group. 16% of the vehicle control group had lung adenomas (average 0.2/mouse) and no lung carcinomas.
NNAL	1 or 2 (S)	Once only	30F(30F)	16	Hecht, Jordon *et al* 1990	The incidence of lung adenomas was:- HD: 90% (3.2 adenomas/mouse); LD: 57% (0.7 adenomas/mouse); Vehicle control: 7% (0.1 adenomas/mouse) NNAL deuterated on the carbinol carbon had substantially the same tumourigenicity.
NNK-1-N-oxide*	1 x 22 (T)	Thrice weekly	25F(25F)	37	Castonguay, Lin *et al* 1983	96% of nitrosamine-treated mice had lung tumours (average 3.6/mouse) of which a quarter were carcinomas. In addition there was one leiomyoma of the uterus in this group. 16% of the vehicle control group had lung adenomas (average 0.2/mouse) and no lung carcinomas.
NNN-1-N-oxide	1 x 22 (S)	Thrice weekly	25F(24F)	37	Castonguay, Lin *et al* 1983	64% of the nitrosamine-treated mice had lung tumours (average 0.8/mouse) of which about a third were carcinomas. 29% of the controls had lung tumours (average 0.4/mouse) including one carcinoma.
3'-hydroxy-NNN	1 x 22 (S)	Thrice weekly	25F(24F)	37	Castonguay, Lin *et al* 1983	48% of the nitrosamine-treated mice had lung tumours (average 0.9/mouse) of which a quarter were carcinomas. Controls as NNN-1-N-oxide above.

TABLE 25 (continued) Carcinogenicity studies of metabolites of tobacco alkaloids or tobacco-specific nitrosamines in female A/J mice[a] dosed intraperitoneally

Metabolite[b]	Dose (mg/mouse) and vehicle[c]	Frequency of dosing	Number per dose group (controls)	Duration of study (weeks	Reference	Observations and comments
4'-hydroxy-NNN	1 x 22 (S)	Thrice weekly	25F(24F)	37	Castonguay, Lin *et al* 1983	76% of the nitrosamine-treated mice had lung tumours (average 1.6/ mouse) of which one third were carcinomas. Controls as NNN-1-N-oxide above.

Notes

(a) The mice were 6-8 weeks old at the start of the study;
(b) NNA is 4-(methylnitrosamino)-4-(3-pyridyl)-butanol. NNAL is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol;
(c) Where the publication gives the dose in molar unit this has been converted to mg (MW: NNA = 207; NNAL = 209; NNK-1-N-oxide = 223; NNN-1-N-oxide = 193; both hydroxy-NNN metabolites = 193. Vehicle: (S) indicates saline; (T) indicates trioctanoin.

TABLE 26 Carcinogenicity studies of metabolites of tobacco-specific nitrosamines in Fischer 344 rats[a] exposed in their drinking water

Metabolite	Dose[b] (mg/rat)	Duration of exposure	Number per dose group	Duration of study (weeks)	Reference	Observations and comments
NNAL (a metabolite of NNK)	Estimated total dose 67	Continuous throughout study	30M(80M)	93	Rivenson, Hoffmann *et al* 1988	The number of rats with tumours was:- NNAL: Lung Adenoma 5; Lung Carcinoma 21; Pancreatic Adenoma 3; Pancreatic Carcinoma 5 Control: Lung Adenoma 3; Lung Carcinoma 3; Pancreatic Adenoma 1; Pancreatic Carcinoma 0 The incidence of other tumours was not significantly increased in the NNAL-treated rats.
NNN-1-N-oxide	Estimated total dose 753(M) 560(F)	Continuous for 36 weeks	12M:12F (12M:12F)	104	Hecht, Young and Maeura, 1983	The number of nitrosamine-treated rats with tumours was:- Benign nasal 8; Malignant nasal 11; Oesophageal papilloma 5; Oesophageal carcinoma 6; Tracheal papilloma 1; Lung adenoma 2; Tongue papilloma 1 No tumours of these types occurred in the controls and the incidence of other tumours was not significantly increased in the rats treated with NNN-1-N-oxide.

Notes

(a) The rats were 6-8 weeks old at the start of the study;
(b) Where the publication gives the dose in molar units this has been converted to mg.

TABLE 27 **Incidence of squamous cell tumours in rats treated with HSV-1 or NQO and snuff. See pages 14 and 43 of text. From Larsson, Johansson *et al*, 1989.**

Group	Number of rats	Carcinoma					Papilloma	
		Ear duct	*Lip*	*Oral cavity*	*Nose*	*Forestomach*	*Lip*	*Forestomach*
I	12	1	1	-	-	-	-	-
II	13	-	-	1	1	-	-	-
III	15	1	-	-	-	-	-	-
IV	12	-	2	1	-	-	-	1
V	12	-	-	2	-	1	1	-
VI	8	-	-	-	-	-	-	-

Notes:- All animals had an artificial lip canal.

Group I = HSV-1 inoculation
Group II = Snuff
Group III = HSV-1 inoculation + snuff
Group IV = NQO
Group V = NQO + snuff
Group VI = Control

TABLE 28 **Effect of tobacco related chemical carcinogens on HSV1, alone or in combination, on the development of oral cancer in the hamster buccal pouch. See page 44 of text. From Park, Min *et al*, 1993.**

Group no	No of pouches per group	No of pouches with tumours	Invasive squamous cell carcinoma
1	20	0*	0
2	20	0	0
3	20	0	0
4	20	0	0
5	20	0	0
6	20	0	0
7	20	4	9
8	18	10	4

Notes:- Two animals died prematurely of encephalitis in group 8.
Groups 1,3,5 and 7 were mock inoculated and then treated topically with mineral oil (Group 1) or with mineral oil containing 1% of NNK(Gp 3), NNN (Gp 5) or BaP (Gp 7).
Groups 2,4,6 and 8 were inoculated with HSV-1 and then treated topically with mineral oil (Group 2) or mineral oil containing 1% of NNK (Gp 4), NNN (Gp 6) or BaP (Gp 8).
No of hamsters per group - 20.

FIGURE 1. Postulated major metabolic pathways of NNK.

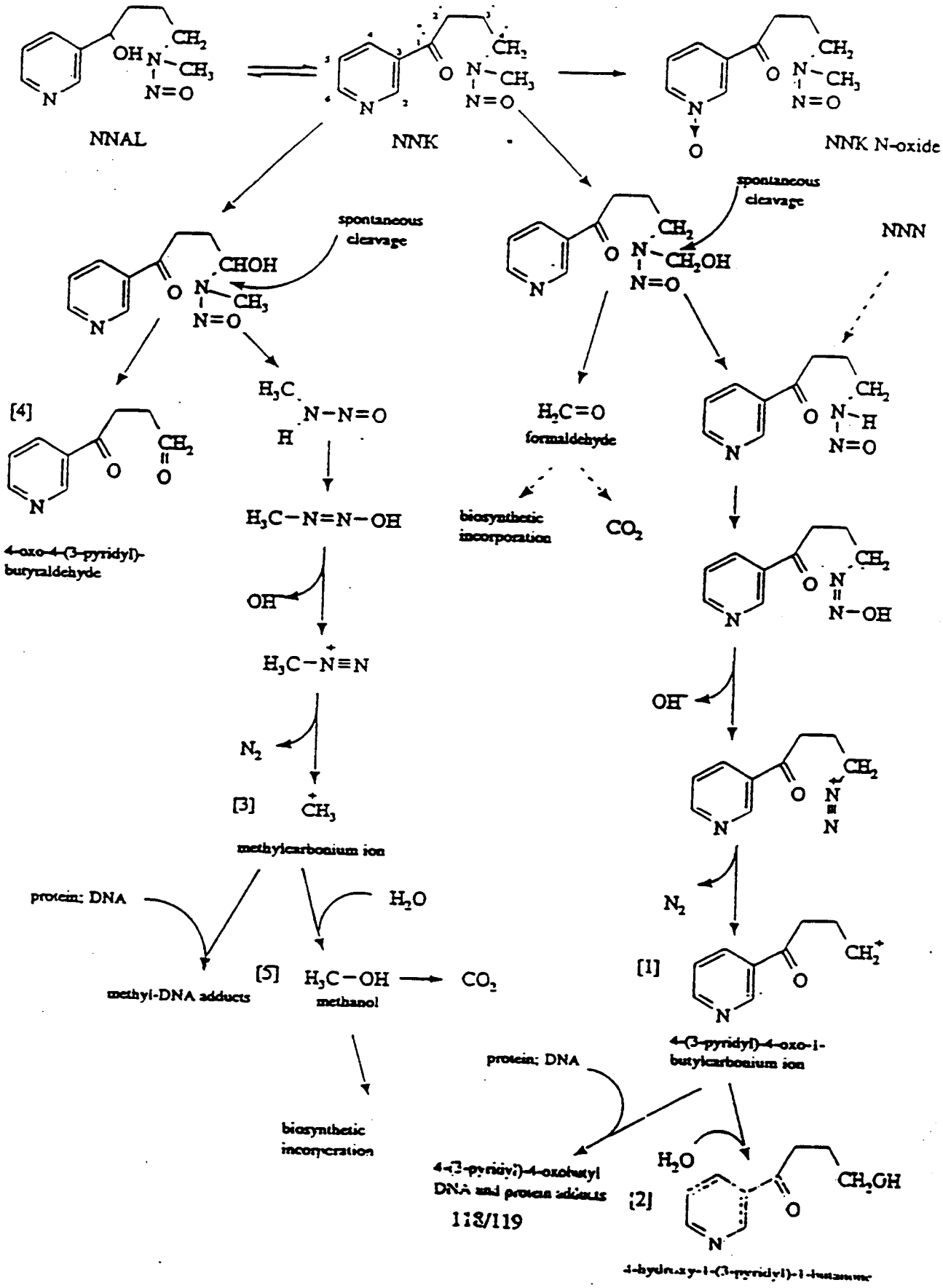
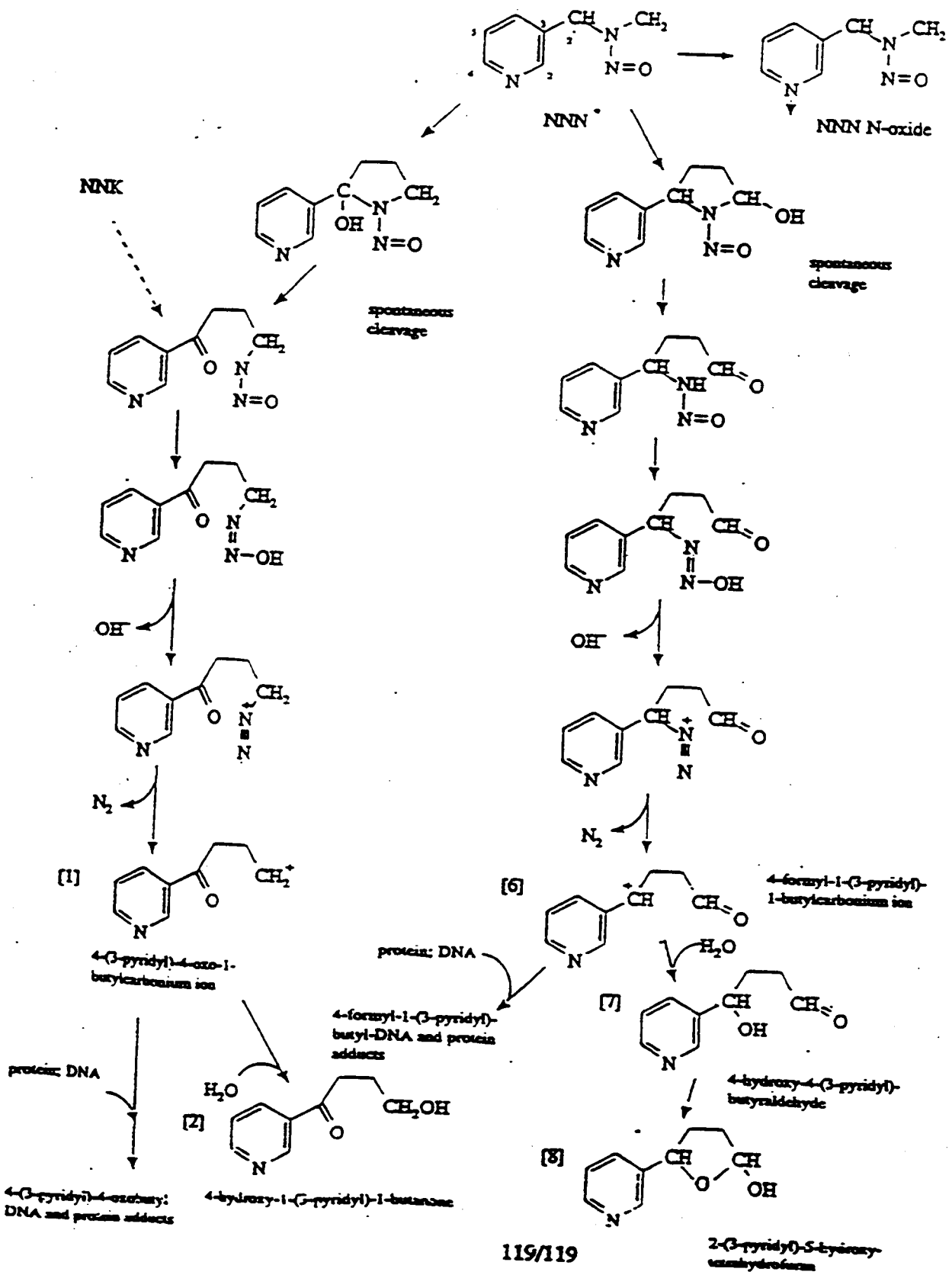


FIGURE 2. Postulated major metabolic pathways of NNN.



REVIEW OF RELEVANT INFORMATION ON EXPERIMENTAL CARCINOGENESIS AND SMOKELESS TOBACCO

Prepared by Torbjörn Malmfors, M.D., Ph.D. for Submission to
National Toxicology Program in Connection with its Review
of Smokeless Tobacco for Possible
Listing in the Ninth Report on Carcinogens

I am Dr. Torbjörn Malmfors. I am currently a consultant toxicologist and principal in the firm Malmfors Consulting AB, Stockholm, Sweden. In 1959 I was awarded the bachelor of medicine degree by the Karolinska Institute, Stockholm, Sweden. In 1965 I defended a doctoral thesis at the Institute and in 1971 I was awarded a doctor of medicine degree by the Institute.

From 1958 to 1962 I was employed as a teaching fellow in the department of histology at the Karolinska Institute. I was then appointed research fellow and in 1965 I was appointed assistant professor. From 1978 to 1984 I was adjunct professor in drug toxicology in the department of pharmacology. I have been a visiting professor in the department of zoology at the University of Melbourne and held the Warren Macdonald fellowship awarded by the National Heart Foundation of Australia. In 1970 I was appointed associated professor of industrial toxicology of the National Institute of Occupational Health in Stockholm and held that position until 1972. From 1972 to 1980 I was employed as head of Toxicology Laboratory of AB Astra in Södertälje, Sweden. I have served as Expert Agree (official expert) in pharmacology and toxicology to the Ministry of Health in France.

I am the author or co-author of approximately 120 publications relating to neurohistology, neuropharmacology, toxicology and risk assessment. I am a member of the Swedish Society of Toxicology and of EUROTOX, a member of the Society of Toxicology (USA), a member of the American Association for the Advancement of Science and a member of the Society for Risk Analysis. In 1984-85 I was president of the Swedish Society of Toxicology. I have also served as Secretary General of the Federation of European Societies of Toxicology. I am presently Director of the International Union of Toxicology and responsible for its Risk Assessment Summer School Programme (RASS).

I have been requested by certain smokeless tobacco manufacturers to review the published experimental data regarding the possible carcinogenicity of smokeless tobacco in experimental animals and to render my professional opinion as to whether smokeless tobacco fulfills the National Toxicology Program's criteria for listing as "reasonably anticipated to be a human carcinogen", which requires in the case of experimental carcinogenesis studies that:

> "There is sufficient evidence of carcinogenicity from studies in experimental animals which indicates there is an increased incidence of malignant and/or a combination of malignant and benign tumors: (1) in multiple species, or at multiple tissue sites, or (2) by multiple routes of exposures, or (3) to an unusual degree with regard to incidence, site or type of tumor or age at onset"

My review of the relevant experimental data is organized as follows:

*Introduction

*Experimental data
- carcinogenicity studies
- studies on the interaction with viruses
- genotoxicity studies

*Evaluation of the relevance of the data

*Conclusion regarding carcinogenicity in experimental animals

Introduction

Smokeless tobacco is a very complex mixture of hundreds of chemical substances (Roberts, 1988). This review, however, is based almost exclusively upon data relating to whole smokeless tobacco, since analyzing the individual components and then evaluating the mixture based upon those results is of doubtful validity in the case of a complex mixture such as smokeless tobacco. The most reliable data in the case of a complex mixture is derived from testing the substance itself rather than its components.

Because of the cost and time required to perform life span animal studies for carcinogenicity, short-term tests, mainly for mutagenicity, are valuable for initial screening to determine which chemicals should be tested further for carcinogenicity. Short-term tests, however, cannot establish a chemical as a cause of a neoplasm because such tests do not have neoplasms as an endpoint.

In evaluating animal studies for carcinogenicity, one should recognize that any conclusion must be based on a demonstration of a statistically significant toxicological effect and a demonstrated dose response relationship. Before one can reach a conclusion that smokeless tobacco is experimentally carcinogenic, it must first be shown, at a minimum, that there is a statistically significant association in animal studies between exposure to smokeless tobacco and malignant neoplasms and that there is a direct dose response relationship i e that the incidence of malignant neoplasms increases with increasing dose.

These evaluation principles are in accordance with the NTP's Criteria for Listing Agents, Substances or Mixtures in the Biennial Report on Carcinogens, which states:

> "Conclusions regarding carcinogenicity in humans or experimental animals are based on scientific judgement, with consideration given to all relevant information. Relevant information includes, but is not limited to dose response, route of exposure, chemical structure, metabolism, pharmacokinetics, sensitive subpopulations, genetic effects, or other data relating to mechanism of action or factors that may be unique to a given substance."

As pointed out above, the evaluation of a complex mixture must be based on the whole mixture, although data on metabolism and pharmacokinetics of mixtures are very difficult to obtain and interpret. Therefore, evaluations regarding possible carcinogenicity in humans based upon studies in experimental animals should rely upon in vivo studies which as closely as possible replicate the use of smokeless tobacco by human beings.

This review summarizes all the published experimental data which are relevant to an evaluation of the possible carcinogenicity of smokeless tobacco in experimental animals in order to be able to judge if smokeless tobacco fulfills the NTP's criteria for listing as "reasonably anticipated to be a human carcinogen".

Experimental data

Carcinogenicity studies

The use of smokeless tobacco by humans involves two major routes of exposure: the local exposure of the oral mucosa to the whole smokeless tobacco and a systemic exposure after absorption by the oral mucosa of various chemicals from the smokeless tobacco and after absorption by the gastrointestinal tract of any parts of the smokeless tobacco which may be swallowed. While it is fairly easy to imitate the systemic exposure by oral administration, it is more difficult to achieve a long-term local exposure. However, various methods have been tried and will be described in this review.

A number of animal studies have been performed in different species and with different types of design including different modes of exposure. The studies are subdivided according to mode of exposure rather than according to species, as the mode of exposure is regarded as more important than the species for the interpretation of the results.

Dietary administration

DiPaolo (1962) fed 34 DBA and 16 C57B1 mice for 15 months and 40 Wistar rats for 18 months with smokeless tobacco in the diet. The dietary concentrations were approximately 5 % for rats and 25% for mice at the beginning of the study decreasing by stages to about 5% at the end of the study. One rat showed a kidney sarcoma and one rat and three DBA mice showed leukemias. Neoplasms are known to occur spontaneously in DBA mice although none was observed in the controls of this particular experiment.

Dunham et al (1974) conducted a feeding study with smokeless tobacco. Approximately 150 mg per day (2.5% of the diet) was given to 20 hamsters for 16 months. No neoplasms were observed in the animals treated with smokeless tobacco only. One animal treated with smokeless tobacco plus lime developed a carcinoid in the pancreas. The authors could not determine whether the carcinoid was spontaneous or induced (Dunham et al 1975).

Homburger et al (1976) examined the possible carcinogenic and co-carcinogenic effects of smokeless tobacco in two different strains of male Syrian hamsters. A total of 500 animals was used. Groups of 50 animals from each strain were given a diet containing 20% smokeless tobacco, a diet

containing 20% cellulose (to reduce the caloric intake, negative control), 5 mg/animal of 20-methylcholanthrene (MC) and normal diet, 0.5 mg/animal of MC and a diet containing 20% cellulose, or 0.5 mg/animal of MC and a diet containing 20% smokeless tobacco for 2 years, respectively. MC was administered 50 times. The animals fed the smokeless tobacco diet alone showed a nearly identical "tumour spectrum" to that of the controls, which means that no carcinogenic effect of smokeless tobacco was observed. As there was no increase in tumour incidence when smokeless tobacco feeding was combined with the administration of MC, the authors also concluded that no co-carcinogenic effect of smokeless tobacco could be demonstrated.

Administration in hamster cheek pouch

The hamster possesses cheek pouches, one on either side of the mouth, which open into the oral cavity and lie underneath the muscles of the cheek, hence the name "cheek pouch". The openings of the pouches lie in the anterior part of the oral cavity and are associated with small salivary glands which produce both serous and mucous secretions. The pouches extend backwards along the oral cavity but do not reach as far as the oropharyngeal junction. Histologically, the epithelium of the cheek pouch is stratified squamous. Functionally, the cheek pouches store half-chewed food which is passed out as needed. Smokeless tobacco, when introduced into the pouch, remains in place for several hours and small amounts are periodically extruded and chewed. Thus, the hamster cheek pouch is a good model to study prolonged exposure of the oral mucosa to smokeless tobacco.

In a study by Peacock, smokeless tobacco or known carcinogens were implanted in the cheek pouch of hamsters. In 21 animals exposed to smokeless tobacco for more than one year, in 11 animals examined within the next 12 months and in the animals surviving 24 months there were no neoplasms of any type (Peacock and Brawley 1959, Peacock et al 1960).

Dunham and Herold (1962) investigated the possible carcinogenicity of smokeless tobacco in a group of 35 hamsters. The smokeless tobacco was incorporated into a beeswax pellet which was inserted into the cheek pouch. The pellet contained 20% smokeless tobacco and was maintained at the site for almost the entire normal life span of about 15-16 months by ligating the neck of the pouch proximal to the pellet. No tumours were observed in the pouch or oral cavity. On the other hand the laboratory carcinogens 7,12-dimethylbenzanthracene (DMBA) and MC incorporated into beeswax in the same way and inserted into the cheek pouch of hamsters, induced carcinomas and sarcomas in the positive controls after 6 months.

A later study by Dunham et al (1966) also using the hamster cheek pouch was similarly negative. Groups of four to seven male and female weanling Syrian golden hamsters received once daily applications of 50 mg of smokeless tobacco, smokeless tobacco and calcium hydroxide (lime), or calcium hydroxide alone in the cheek pouch five days per week for up to 99 weeks. No oral neoplasms were observed in any group.

Homburger (1971) also investigated the possible carcinogenic properties of smokeless tobacco by using the hamster cheek pouch as well as the oral mucosa. He immobilised the animals' heads for 30 minutes each day, which allowed smokeless tobacco to be placed in a stainless steel webbing attached to a stainless steel bit to be applied with an automatic cartridge filler to the gingivolingual area, including the upper part of the cheek pouch. A total of 84 male and female Syrian golden hamsters were exposed for up to 51 weeks. The epithelium of the lip of the oral cavity and of the cheek pouch of the animals exposed to smokeless tobacco showed only minor changes compared to controls. One benign tumour (papilloma) was found in each of the groups exposed to smokeless tobacco and cotton (control group) respectively. No carcinomas were found in the oral mucosa. In contrast, carcinomas of the oral mucosa were found after a similar exposure in the same area to DMBA (three squamous cell carcinomas in the pouch and two in the oral cavity).

In another study with male hamsters, 70 mg of finely powdered smokeless tobacco or 50 to 100 mg of coarser tobacco were introduced daily for 20 weeks into the cheek pouch of groups of 20 animals. The experiment was terminated after 20 weeks. While no significant pathological changes were observed in these animals there was a slight diminution of mitotic activity and an increase in Langerhan's cells (Shklar et al 1985).

The application of 2 g of smokeless tobacco to the blind end of the right cheek pouch of a group of 8 male hamsters, 5 days a week for 6 months (terminated at 6 months) resulted in no local proliferative leisions or neoplasms. Hyperplasia of the cheek pouch epithelium was noted (Worawongvasu et al 1991).

Topical application

Although the cheek pouch model is a good way to study both the local and systemic effects after local exposure, it is limited to species with that special anatomy i e hamsters. In order to perform studies in rats with local

exposure, Hecht et al (1986) devised a protocol in which groups of rats were treated by application to the oral cavity of aqueous extracts of smokeless tobacco. The extracts were applied by swabbing the oral cavity and lips usually twice daily for 131 weeks with a cotton swab to which 0.5 ml solution had been added. The identification of certain tobacco-specific nitrosamines (TSNAs) in smokeless tobacco has raised questions about their possible effects as components of smokeless tobacco. Therefore, Hecht et al exposed a second group to smokeless tobacco aqueous extract enriched with NNN (N'-nitrosonornicotine) and NNK [4-(methylnitrosamino)-1- (3-pyridyl)-1-butanone] up to a concentration ten times higher than that in the plain smokeless tobacco extract. A third group received NNN and NNK in water at the concentrations added to the extract. The controls were swabbed with water.

No oral neoplasms were observed in the 30 animals treated with the aqueous extract of smokeless tobacco. In the 30 animals treated with NNN and NNK in water, 8 oral papillomas were found, which was statstically significant. However, in the 30 animals treated with NNN and NNK in the aqueous smokeless tobacco extracts, only 3 oral papillomas were found. Hecht et al concluded that "(t)he lack of oral tumours in the animals treated with snuff extract indicates that this mixture is not tumorigenic in the rat oral cavity, when the swabbing protocol was used". The lower number of oral neoplasms in the animals exposed to smokeless tobacco aqueous extract enriched with NNN and NNK, while not statistically significant, suggests, according to the authors, that smokeless tobacco has an apparent "inhibitory effect" on the metabolic activation of NNN and NNK.

Using a primate species as the experimental animal, Smith placed smokeless tobacco in the cheeks of 12 rhesus monkeys for a total of up to seven years using a variety of techniques. No neoplasms were produced (Smith et al, 1970).

Administration into a surgically created canal in the lower lip of rats

Hirsch and Thilander (1981) developed a rat model that sought to simulate human "dipping" in experimental animals. Using surgical techniques, they created an artificial canal in the lower lip of young adult rats which was open at both ends and which was lined internally by mucosal epithelium and externally by skin from the lip. Tobacco or similar solid products could then be inserted and replaced readily as required by the experimental protocol. The operation initially caused a marked inflammatory reaction. As far as could be ascertained, the tissue reaction at the site of surgery was allowed to

heal before being used for experimental purposes. The test material was injected from the lip side into the artificial canal by a plastic syringe until excess snuff was pressed out through the buccal opening, ensuring complete filling of the canal. The authors found that this model could accomodate approximately 0.29 g of powdered smokeless tobacco which corresponds to a mean dose of 1 g/kg body weight (approximately 5 times the amount a human might use). The inserted material was retained for 5 to 8 hours and was accompanied by "hypersalivation" and a marked increase of blood nicotine. After a twice daily application of 0.2 to 0.4 g of powdered smokeless tobacco for 9 months the epithelium of the canal was mildly to moderately hyperplastic and the adjacent connective tissue exhibited an inflammatory reaction which varied in degree from mild to severe. No tumours were reported (Hirsch and Thilander 1981).

The lip canal model was used by Hirsch and Johansson (1983) to study the effects of long-term application of smokeless tobacco. Rats received standard smokeless tobacco (42 animals) or highly alkaline smokeless tobacco (ten animals) twice daily, 5 days a week for 9-22 months. Animals with identical canals, but not treated further, served as controls (15 animals). A complete post-mortem examination was performed.

Animals exposed to standard smokeless tobacco after 9-12 months (26 animals) developed a mild to moderate hyperplasia of the epithelium, hyperorthokeratosis and acanthosis. The oral mucosa of rats exposed for 18-22 months (16 animals) showed about the same changes. In a few rats, severe dysplastic changes developed in the crevicular epithelium (the epithelium of the gingival sulcus of the lower incisors). Rats exposed to alkaline smokeless tobacco differed little from the other ones exposed. The lips of the control animals mostly showed mildly hyperplastic epithelium which was covered with orthokeratin. One macroscopic tumour was observed in a rat treated with standard smokeless tobacco after 8.5 months. It was situated on the left side of the oral cavity, extending from the incisors and involving both the upper and lower jaws. The tumour was a moderately well-differentiated squamous cell carcinoma invading the bone. The authors could not, however, determine whether the neoplasm was "spontaneous or induced". There were few pathological findings outside the oral cavity.

Using the lip canal technique Hirsch et al (1984) observed moderately well-differentiated squamous cell carcinomas in two animals infected with herpes simplex virus type 1 (HSV-1) and exposed to smokeless tobacco twice daily, 5 days peer week for 18 months. No tumours were detected in non-infected animals exposed to smokeless tobacco (10 animals), HSV-1 infected animals not exposed to smokeless tobacco (7 animals), or untreated controls (10

animals), all subjected to lip canal surgery. The HSV-1 infected rats exposed to smokeless tobacco showed a significantly higher incidence of malignant tumours than the control animals and the animals only infected with HSV-1. An increased fibrosis was observed in the treated animals compared to the controls.

In order to further study the dysplastic changes observed earlier (Hirsch and Johansson 1983) , Hirsch et al (1986) exposed 30 rats to smokeless tobacco in the same way as in the earlier study for 13 months. In ten rats examined immediately after the exposure similar changes as before were found. These changes were markedly reduced or absent in rats exposed to smokeless tobacco and examined after a recovery period of 1 or 4 months. It was concluded that the epithelial changes induced by smokeless tobacco were reversible. The crevicular epithelium appeared to be more sensitive to smokeless tobacco exposure than that of other parts of the oral cavity. This is probably due to the short distance between the lip canal and the incisors. Furthermore, it seems likely that smokeless tobacco is more or less constantly retained in the gingical sulcus, resulting in a longer exposure time in comparison with other areas of the oral cavity. However, the subepithelial connective tissue exhibited extensive fibrosis, which did not recover. The authors state that the three tumours they have observed (see Hirsch and Johansson 1983, and Hirsch et al 1984) after 9 months (one tumour) and 18 months (2 tumours) most likely originated from the crevicular epithelium and not from the squamous epithelium of the lip canal.

The lip canal technique was also employed to explore the possible carcinogenicity of smokeless tobacco in the oral cavity of F344 rats by Hecht et al (1986). The results were published together with the results of studies with topical application of aqueous extracts of smokeless tobacco. Groups of rats were treated by administration in the lip canal of either 50 mg smokeless tobacco (32 animals), the residue after aqueous extraction of smokeless tobacco (21 animals) or smokeless tobacco enriched with its own aqueous extract (32 animals) 5 times weekly for 116 weeks. A group of 10 rats, which were subjected only to surgery, served as controls. Two tumours, one of which was malignant, developed in the epithelium of the lip canal and there was one papilloma of the hard palate in the group treated with whole smokeless tobacco. In the group treated with extracted smokeless tobacco residue one rat had a papilloma of the tongue and another rat a papilloma of the hard palate. One animal in the group treated with enriched smokeless tobacco developed a papilloma of the floor of the mouth. None of these observations were statistically significant compared to the controls.

Administration into a surgically created canal in the lower lip of rats in combination with other chemicals

In order to further study the possible carcinogenic effects of smokeless tobacco, particularly any promotion effect, a few studies have been performed in which the administration of smokeless tobacco in the lip canal has been combined with local exposure to 4-nitroquinoline-N-oxide (NQO) or 7,12-dimethylbenz(a)anthracene (DMBA), both compounds defined as initiators.

Johansson et al (1989) combined exposure to smokeless tobacco in the lip canal with exposure to NQO painted on the hard palate. One hundred and fifty Sprague-Dawley rats had a lip canal created surgically in the lower lip.

The rats were divided into 5 groups, up to 30 animals in each. One month after surgery the groups were treated in the following way:

Group I.	Smokeless tobacco, 5 days a week up to 104 weeks
Group II.	Propylene glycol for 4 weeks
Group III.	NQO, 3 times a week for the first 4 weeks of the experiment
Group IV.	NQO for 4 weeks and then smokeless tobacco for 104 weeks
Group V.	Cotton pellet (untreated control)

The lip canal was filled with at least 100 mg smokeless tobacco twice daily. NQO was dissolved in propylene glycol to a concentration of 0.5% and about 0.13 mg of NQO was applied.

All remaining rats were terminated 108 weeks after start of treatment.

There was no significant difference in mean survival time between the groups. The groups treated with smokeless tobacco had a slower weight gain than the other groups and the lower body weight remained statistically different from the controls during the whole experiment due to a lower food intake.

Tumours and hyperplastic leisons were found in all groups. The observed incidence per group in the oral cavity:

Group	I	II	III	IV	V
Number of examined animals	29	28	29	28	29
Type of lesion and location					
Carcinoma					
Lip (in situ)			1	(1)	
Hard palate (in situ)	2(1)		2	4	
Tongue			2	1	
Papilloma					
Lip	1				
Hard palate	1				
Tongue			2	1	
Sarcoma					
Lip	2			3	
Hyperplasia					
Lip, lip canal	24	6	4	25	10
Hard palate	18	7	7	14	2
Dysplasia					
Lip, lip canal	10		4	12	
Hard palate	5			7	

Outside the oral cavity a few tumours were found in various organs. The same type of tumour was not found in more than one or two animals per group except in Group I where four animals showed a malignant lymphoma.

There were a total of 23 and 22 tumours in Groups I and IV, respectively. The difference in total tumour incidence between Groups I and IV and Groups II and III was reported by the authors to be statistically significant but not the difference between any of the groups for individual types of tumours. There was no difference between Group I and Group IV and thus it was concluded that smokeless tobacco had no promoting capability when the rats were initiated with NQO.

Larsson et al (1989) did a similar experiment with NQO but combined it with an investigation of the possible interaction of smokeless tobacco with HSV-1 virus. Fiftyfive Sprague-Dawley (SD) rats and 30 Lewis (L) rats had a lip canal created surgically in the lower lip.

The rats were divided into 6 groups, up to 15 animals in each. Ten days after surgery the groups were treated in the following way:

Group I	SD	HSV-1, once a month
Group II	SD	Smokeless tobacco, 5 days a week for up to 30 months
Group III	SD	HSV-1 and smokeless tobacco
Group IV	L	NQO, once weekly for the first 5 weeks
Group V	L	NQO first and then smokeless tobacco
Group VI	SD	Propylene glycol on a cotton swab, control

The lip canal was filled with at least 200 mg smokeless tobacco twice daily. NQO was dissolved in propylene glycol to a concentration of 0.5% and 0.05 ml of the solution, about 0.25 mg of NQO was absorbed in a cotton swab, which was put in the test canal for 24 hours. The animals were infected with HSV-1 in a suspension, which was absorbed in a cotton swab. The swab was placed in the lip canal after scarifying the surface with a needle.

The animals were killed when moribund after 16-30 months.

The Lewis rats survived 6 months longer than the Sprague-Dawley rats. The groups treated with smokeless tobacco had a slower weight gain than the other groups and the lower body weight remained statistically different from the controls during the whole experiment due to a lower food intake.

Tumours and hyperplastic leisons were found in all groups. The observed incidence per group in the oral cavity:

Group	I	II	III	IV	V	VI
Number of examined animals	12	13	15	12	12	8
Type of lesion and location						
Carcinoma						
Lip	1			2		
Crevicular epithelium		1		1	2	
Papilloma						
Lip					1	
Hyperplasia						
Lip, lip canal	2	5	5	2	10	2
Crevicular epithelium	5	11	14	5	8	1
Dysplasia						
Lip					2	
Crevicular epithelium			2		2	

Outside the oral cavity a few tumours were found in various organs. The same type of tumour was not found in more than one or two animals per group except in Group V where three animals showed a pheochromo-cytoma in the adrenal gland.

There were a total of 3, 4, 13, 8, 12, and1 animals with tumours, both malignant and benign, in Groups I, II, III, IV, V and VI, respectively. The total number of tumours was reported by the authors to be statistically significantly higher in Group III as compared to Groups I, II, and VI but not the difference between any of the groups for individual types of tumours. There was no difference between Group IV and Group V and thus it was concluded that smokeless tobacco had no promoting capability when the rats were initiated with NQO.

Johansson et al (1991) combined exposure to smokeless tobacco in the lip canal with exposure to NQO or DMBA on a cotton pellet placed in the "pouch". Two hundred and thirty male Sprague-Dawley rats had a lip canal created surgically in the lower lip.

The rats were divided into groups, up to 40 animals in each. Two weeks after surgery the groups were treated in the following way:

Group I. DMBA, 3 times a week for the first 4 weeks, then saline
Group II. DMBA first and then smokeless tobacco for up to 104 weeks
Group III. Smokeless tobacco, 5 days a week for up to 104 weeks
Group IV. NQO, 3 times a week for the first 4 weeks, then saline
Group V. NQO first and then smokeless tobacco for up to100 weeks
Group VI. Cotton pellet dipped in saline(control)

The lip canal was filled with approximately 150-200 mg smokeless tobacco twice daily. NQO was dissolved in propylene glycol to a concentration of 0.5% and DMBA was dissolved in mineral oil to a concentration of 0.1%. NQO or DMBA. The rats received approximately 70 mg of the solution at each application.

The rats were killed when moribund, when they developed lip tumours, or after 104 weeks of treatment.

The difference in mean survival time between Group V and all of the other groups was reported by the authors to be significant. The groups treated with smokeless tobacco had a slower weight gain than the other groups and

the lower body weight was reported by the authors to remain statistically different from the controls during the whole experiment.

Tumours and hyperplastic leisons were found in all groups. The observed incidence per group in the oral cavity:

Group	I	II	III	IV	V	VI
Number of examined animals	40	40	38	40	38	30
Type of lesion and location						
Carcinoma						
Lip		1		1	1	
Palate		2	3	6	5	
Buccal mucosa				1		
Papilloma						
Lip			2	1	2	
Sarcoma						
Lip		9	10	1	25	1

Outside the oral cavity a few tumours were found in various organs. The same type of tumour was not found in more than one or two animals per group except in Group I where three animals showed renal cell tumours.

There was a total of 14, 28, 24, 20, 40 and 5 tumours in Groups I, II, III, IV, V and VI, respectively. The difference in incidence of sarcomas between Group V and Groups I, II, IV, and VI and the difference between Groups II and III and Groups I, IV, and VI was reported by the authors to be statistically significant but not the difference between any of the groups for any other individual types of tumour. The sarcomas in Group V developed as early as after 29 weeks. The tumours were large. The majority of tumours were undifferentiated spindle cell sarcomas of nonepithelial mesenchymal nature. It was concluded that the incidence of sarcomas after exposure to smokeless tobacco was higher when the rats were initiated with NQO but not with DMBA.

Studies on the possible interaction between smokeless tobacco and viruses

Different viruses have been implicated in mammalian carcinogenesis and herpes viruses constitute a large group of viruses which infect most mammalian species. Thus, it is of interest to study the possible interaction between chemicals or mixtures of chemicals to which humans are exposed

Two of the studies have been briefly reviewed above(Hirsch et al, 1984 and Larsson et al, 1989). Park et al (1986) innoculated the cheek pouches of Syrian hamsters with HSV once a month for six months. Smokeless tobacco was inserted twice daily into both pouches of half of the animals. At the conclusion of the experiment, the pouches of all of the animals appeared grossly normal. Histological examination revealed that none of the animals exposed to smokeless tobacco alone or HSV alone developed any cancers. Epithelial dysplasia and squamous cell carcinomas were observed in over half the animals exposed to HSV in combination with smokeless tobacco. The authors concluded that "our data suggest that smokeless tobacco alone does not induce precancerous or neoplastic changes in hamster buccal pouches". As to the respective roles that smokeless tobacco and HSV might have played in the development of the neoplasms observed, the authors stated, "since repeated HSV infection alone induced precancerous changes and simulated snuff dipping alone did not cause the dysplastic changes in the pouch tissues, HSV may be more responsible for the development of neoplastic changes". Although there has been speculation regarding the mechanisms that might have produced this result, there are as yet no clear answers.

Studies involving other possible interactions with HSV underscore the complexities relating to the possible carcinogenic activity of viruses. One study reported enhanced oncogenic activity of HSV associated with exposure to UV-light (Burns and Murray, 1981) and another has reported increased cancer frequency when HSV was combined with cortisone or with trauma created with a brush (Chen and Swen, 1987).

There is little doubt that HSV infected cells in vitro can be transformed into potentially malignant cells by agents that suppress the cytolytic effect of the virus such as UV-light radiation and chemical carcinogens. This suppression is thought to be achieved by interference with the cellular synthesis of proteins essential for the growth of the virus while leaving intact the expression of the viral genome. In vivo studies do not, however, present such a clear picture. The experimental results are inconclusive.

Mutagenicity

As has been underscored in the introduction, short-term tests, mainly for mutagenicity, cannot establish a chemical as a cause of a neoplasm because such tests do not have neoplasms as an endpoint. Furthermore smokeless tobacco in its whole form is unsuitable for mutagenicity testing. Only extracts can be used, which are not completely representative of whole smokeless

tobacco. Positive results have been obtained when aqueous extracts were tested in bacterial mutagenicity tests and when organic or aqueous extracts were tested against cultures of human cells in vitro. The significance of these results is not clear.

Evaluation of the reviewed data

In order to investigate the possible effects chemicals or mixtures of chemicals might have on human health, studies in vitro or in animals must be used as surrogates since the ability to obtain information directly from human beings is very limited. Experiments can hardly be performed and clinical and epidemiological observations have significant shortcomings.

However, the surrogate information must be critically and thoroughly evaluated from a scientific point of view. While there is evidence of sufficient similarities between animals and humans to warrant animals being used as surrogates, there is enough knowledge about significant differences to make a careful evaluation necessary.

There are no standardized guidelines either on how the data should be evaluated or on when and how the surrogate data can be extrapolated to human beings. Evaluations have to be done on a case-by-case basis taking into consideration the studied chemical(s), the test system and the results obtained.

Since a true extrapolation is involved and there are significant knowledge gaps regarding similarities and differences between the animal species used and human beings, judgements must be made to a large and variable extent. The judgements have to be based upon generally accepted scientific, toxicological principles and acquired experience.

The evaluation process has two major aims. First to review the studies performed and evaluate the significance and the validity of the overall results and conclusions. Second to evaluate the relevance and importance of the results and conclusions to human beings.

In the present case there are several aspects which must be given special consideration:

* Smokeless tobacco is a very complex mixture.

* The special mode of exposure in humans is difficult to imitate in experimental animals, which has lead to the use of different administration methods for which there is limited experience.

* The studies available have been performed during a rather long time period (more than 30 years) during which the state-of-the-art of toxicological studies has changed considerably.

* There is a limited amount of information on the toxicokinetics and toxicometabolism of smokeless tobacco both in animals and human beings . which limits the possibility of comparisons between species.

* Due to lack of information on any mode of action, possible interactions between different components of smokeless tobacco, possible interactions with other chemicals or mixtures (e g food), or possible interactions with micro-organisms (e g viruses) are very difficult to evaluate.

The quality and quantity of the information contained in the study documents varies. They are all published in scientific journals but some of them are not sufficiently comprehensive to be evaluated in detail. This is particularly true for the older publications.

None of the studies has been performed according to Good Laboratory Practices (GLP) but later studies ought to have adopted the principles of GLP. However, even if GLP is considered important particularly in the case of studies with no finding of any effects, older studies, briefly described, and not performed according to GLP cannot be ignored but must be evaluated on their own merits at the time of their publication.

The first aim of this evaluation will be approached with the second aim in mind. The mode of exposure is considered a suitable starting point for the evaluation.

Systemic exposure has been achieved both by dietary administration and by various modes of exposure in the oral cavity. It seems reasonable to assume that the most complete and extensive exposure has been achieved by the former. There are at least two studies (DiPaolo, 1962 and Homburger, 1976) which have provided useful information. They include sufficient numbers of animals of three different species, exposed to high dose levels for almost a life-span period. There was no increase in any type of neoplasms and hence no evidence of any systemic carcinogenic effect of smokeless tobacco in the species used. Although the studies are old, from the pre-GLP time period, and poorly reported, they must be considered valid and important in the overall evaluation of any possible systemic carcinogenesis.

None of the studies employing local administration in the oral cavity of the animal has revealed any statistically significant increase of any individual type of neoplasm in organs exposed systemically. In a few studies (Johansson et al, 1989 and Larsson et al, 1989) a statistically significant

increase in overall tumour incidence was reported. However, that does not necessarily support a theory of a direct cause-effect relationship since it is inconsistant with the principle of target specificity of carcinogens and since an increase in the overall incidence, if real, probably would be related to noncarcinogenic effects.

None of the various modes of exposure in the oral cavity replicates exactly the usage by human beings. The hamster cheek pouch model has been used by several investigators. In no study has there been any evidence of any carcinogenesis though it has been shown that the cheek pouch epithelium is sensitive to experimental carcinogens like DMBA.

Similar negative results have been obtained after application of smokeless tobacco and DMBA to the oral mucosa of hamsters, which have been temporarily immobilized allowing a prolonged exposure with the help of special equipment.

All of the studies in hamsters are somewhat old and the design, particularly the mode of exposure, specialized. However, a substantial local exposure of the oral mucosa has obviously been achieved without any neoplasm formation, while positive controls have produced adequate responses.

In rats there are no studies with whole smokeless tobacco applied to an intact oral cavity as that probably is impossible to achieve. Besides exposure of aqueous extracts of smokeless tobacco to the oral mucosa, exposure to whole smokeless tobacco has been achieved by administration into a surgically created canal in the lower lip. While this mode of exposure allows a long-lasting exposure with high doses to the oral epithelium, the surgical procedure induces a long-lasting inflammatory reaction with subsequent fibrosis.

Despite this extensive local exposure no study has revealed any statistically significant incidence of any individual type of neoplasm derived from the oral epithelium. Neither has any promotion effect on such tumours been observed when smokeless tobacco has been administered following exposure to known experimental initiating carcinogens like NQO.

The only statistically significant effect on an individual type of neoplasm observed was an increased incidence of sarcomas in the lower lip of rats given smokeless tobacco with or without DMBA or NQO in one study. Three other studies with similar design and exposure did not reveal any statistically significant increased incidence of lip sarcomas.

Thus, it can be concluded that the only positive results of the reviewed, experimental studies which are sufficiently significant to be considered as possible surrogate information for what could happen in human beings is the

finding of sarcomas in the lower lip of rats exposed to smokeless tobacco administered into a surgically created canal. This result then has to be evaluated with regard to its relevance and importance to human beings.

Animal models are utilized in carcinogenicity studies on the assumption that any response to a chemical or chemical mixtures in test animals will be similar to the response in humans. Therefore, the results of animal studies must be considered relevant to a carcinogenicity evaluation in humans unless there is reason to discount them.

In the case of smokeless tobacco the use of a surgically created canal in the lower lip of rats in order to make prolonged exposure feasible causes a marked reaction in the submucosal connective tissue and a subsequent fibrosis. In rodents, such as rats, such reactions are known to preceed the formation of sarcomas (Grasso et al 1991) while a similar pattern of events in human beings has not been encountered.

Thus, there are sufficient reasons to question the relevance and importance to human beings of the sarcomas observed in the lower lip of rats after long-term exposure to smokeless tobacco administered in a surgically created canal.

Conclusion regarding carcinogenicity in experimental animals

There has been a substantial number of animal studies performed to investigate the possible carcinogenicity of smokeless tobacco. Studies have been performed in mice, hamsters, rats and monkeys. Different modes of exposure have been employed for a life span period in most instances. A maximally possible amount of smokeless tobacco has been administered in most experiments both systemically and locally. The animal strains used and the most relevant sites have all been shown to be sensitive to various experimental carcinogens.

The studies have been performed over a long period during which the standards for carcinogenicity studies have changed considerably. Even if there are technical insufficiences particularly in the reporting of earlier studies they have to be taken into account in the overall evaluation.

My overall evaluation of the animal studies is that there are no results which are sufficiently significant, valid, or relevant to human beings according to scientific principles, to justify a conclusion under the criteria established by NTP that smokeless tobacco is "reasonably anticipated to be a human carcinogen".

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